DETERMINING FACTORS OF PREFERENTIAL HOST SELECTION BY *Aedes aegypti*

By

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By

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Abstract of Dissertation Presented to the Graduate School
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DETERMINING FACTORS OF PREFERENTIAL HOST SELECTION BY *Aedes aegypti*

By

Karen Elaine McKenzie

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Chair: Jerry F. Butler
Major Department: Entomology and Nematology

This study attempts to determine whether colony reared *Aedes aegypti* show human host preference selecting a blood meal and compares different formulations of artificial host media to determine relative attractiveness. Human Subject 8005 was significantly less attractive than Standard 3776 ($\alpha = 0.05$, $p = 0.04$) and Subject 8007 was significantly more attractive than 3776 ($\alpha = 0.05$, $p = 0.03$). All other subjects were statistically the same as 3776. Gas chromatography (GC) of two subjects showed unique peaks.

There was a significant difference between Media 3776 (blood, agar and BSS® Plus) and 3777 (agar and BSS® Part I) as well as Medium 3778 (agar and BSS® Plus); but none between Media 3776 and 3779 (blood, agar and BSS® Part I) ($\alpha = 0.05$, $p = 0.03$, $p = 0.01$, $p = 0.40$). There also was a significant difference between Media 3777 and 3779 ($\alpha = 0.01$, $p = 0.007$) and Media 3778 and 3779 ($\alpha = 0.005$, $p = 0.003$); but none between Media 3777 and 3778 ($p = 0.23$).

Chemical studies were undertaken to determine attractant qualities of certain compounds found naturally on human skin or in cosmetic beauty products. In the first set, treatment 4786 significantly differed ($\alpha = 0.05, p = 0.04$) from 3776 (no treatment). In set two, six of nine chemical treatments were significantly different from 3777 (no treatment) ($\alpha = 0.01, p = 0.003$, $\alpha = 0.10, p = 0.08$, $\alpha = 0.001, p = 0.0001$, $\alpha = 0.10, p = 0.07$, $\alpha = 0.10, p = 0.08$, $\alpha = 0.05, p = 0.02$). In set three, treatments 3906 and 3967 were significantly different than 3778 (no treatment) ($\alpha = 0.05, p = 0.02, p = 0.01$). In set four, treatment 4792 was significantly different than 3778 (no treatment) ($\alpha = 0.05, p = 0.02$). Data from cholesterol trials varied. In trials 1 and 5 cholesterol was attractive ($\alpha = 0.10, p = 0.09$; $\alpha = 0.10, p = 0.08, p = 0.08$) and in trials 3 and 4 cholesterol was repellent ($\alpha = 0.10, p = 0.04$; $\alpha = 0.10, p = 0.07$).

CHAPTER 1
MOSQUITO FEEDING AND HOST ATTRACTION

History of *Aedes aegypti*

Mosquitoes are of biological and economical importance to man because of their role in the spread of diseases; and because of the millions of dollars of losses they cause each year in the livestock industry (Ebeling 1975). Patrick Manson first confirmed mosquito vectored disease in 1877 when he discovered larval filarial worms (*Wucheria bancrofti* (Cobbold)) in *Culex quinquefasciatus* Say adults (Lehane 1991). Mosquito feeding can directly affect both human and animal health.

When mosquitoes feed, pathogens of some of the most deadly diseases can be transmitted directly by the feeding process; or through waste excreted on the skin during feeding. Mosquitoes are the most important vectors of many human diseases (including filariasis, yellow fever, dengue fever, elephantiasis, viral encephalitis, West Nile Virus, and malaria) (Harwood and James 1979, Jarial 2001). Malaria infects 300-500 million people per year and kills more than 1.5 million people per year. Filariasis infects 12 million people per year. Dengue fever infects 50 million people per year (Adams 1999). Because of mosquitoes' devastating effect on humans, extensive research has been done on controlling these disease-spreading insects (Friend and Smith 1977).

The sheer nuisance and pest factor status of mosquitoes has prompted funding for much of the research. In South Florida near the Florida Everglades (and even near theme parks), prime tourist season is also prime mosquito season. At certain times of the year, theme parks are often closed before dusk to help tourists avoid infection. Mosquitoes can be so numerous in the Everglades that thousands land on a human wearing repellent, even during the day. During active feeding times, it is nearly impossible to enjoy being outside when mosquitoes are hunting for blood. At night (a prime feeding time for some species), mosquitoes rely on senses other than vision (Taubes 1998), and can travel as far as 106 km to find food (Service 1993).

Aedes aegypti (subgenus *Stegomyia*) as described by Linnaeus in 1762 has been living in the United States for centuries; and by the eighteenth century was considered the most common mosquito to bite during the day in tropical and subtropical countries. These mosquitoes populated the world by hitching rides on slave ships from Africa; an association that served to domesticate *Ae. aegypti* (L.). Given a choice, they prefer to live indoors (Adams 1999). This close association with man gave mosquitoes the opportunity to transmit diseases, such as yellow fever, dengue, and dengue hemorrhagic fever. Living indoors also increased their life span and feeding ability. The females evolved the ability to bite quite gently and thus to avoid detection while feeding. They adapted to their environment so efficiently that they can use blood for an energy source. An increase in blood meals per gonotrophic cycle increases their vector potential (Adams 1999). Also, because of its nervous habits, even the slightest disturbance can disrupt a meal, causing the mosquito to feed on more than one host to complete one blood meal. This multiple feeding is another reason that *Ae. aegypti* makes such an efficient vector. Yet another reason is their ability to transmit disease when probing for blood, so that actual feeding is not necessary to transfer a virus (Taubes 1998).

Although not the top disease transmitting species, *Ae. aegypti* has a longer history of synanthropy than the top genus *(Anopheles)* that vectors human disease (Laird 1989). *Aedes aegypti* originated in Africa where its larval habitat was predominantly tree holes; but when man came down from his arboreal habitat, *Ae. aegypti* came too (Service 1989). Presently, *Ae. aegypti* larvae develop in water-filled containers inadvertently provided by humans; and thus has become almost completely dependent on humans for its larval habitat (Laird 1989).

Mosquitoes seek a blood meal to get the protein needed for oviposition (Adams 1999). Only female mosquitoes feed on blood; males feed on plant sugars or honeydew. Some autogenous species of mosquito can produce the first batch of eggs without taking a blood meal, but successive egg batches will only develop after acquisition of a blood meal from a vertebrate host. These species can transmit disease, but have a lower vectorial capacity than species that must feed prior to all egg batches. Female mosquitoes also take nectar, which is used as an energy source for metabolism and flight. Mosquito behavior has evolved in a manner that offers optimal success in searching for blood meals. As a direct result of this evolutionary advance, mosquitoes are thriving because more eggs are produced with larger blood meals. For example, if an *Ae. aegypti* takes a 1- mg blood meal it will develop approximately 10 eggs; increasing that blood meal by 2- mg, however, increases egg development to an astonishing 83 eggs (Adams 1999).

Importance of *Aedes aegypti* as a Disease Vector

Several attributes are required for a mosquito to be a successful vector. First, the causal organism must multiply within the mosquito, thus increasing the number present in the salivary glands and making it possible for the mosquito to pass it on by injecting infected salivary fluid on or in the next host. Second, the causal organism must be able to multiply in the fresh host, so that there will be enough of it present for another mosquito to pick up the organism when it feeds on the host. Not all mosquitoes have the ability to culture a disease organism that is transmitted by another mosquito. For example, Eastern equine encephalitis (EEE) is a naturally occurring virus in Florida transmitted by ornithophilic mosquitoes, which may occasionally bite horses and humans. Approximately 150 horses die each year from EEE (as well as a few humans, but human death is not a common occurrence). Another example is West Nile Virus, which has killed humans in the United States in the past few years. It is a virus that is normally found in birds and is therefore carried by mosquitoes that do not typically feed on other animals. If *Ae. aegypti* or *Aedes albopictus* (two anthropophilic species) could effectively carry these viruses, probably many more humans would be infected each year (Taubes 1998).

Dengue is largely an urban disease, found primarily in Southeast Asia and the Western Pacific; but it also now occurs in the Caribbean. The major vector is *Ae.aegypti*, with *Ae. albopictus* also playing a part in some areas of the world. Earliest records of dengue are from 1779 in the Mediterranean and 1780 in Philadelphia. During the eighteenth and nineteenth centuries, outbreaks occurred in newly settled areas, as new urbanization created places for the vector to establish itself. Since the 1920s dengue epidemics have not been important in most parts of the United States, with the exception of outbreaks in Florida in the 1940s. This is probably attributable to the use of window screens, air conditioners, good public sanitation, televisions and telephones (which keep people indoors) (Taubes 1998). Urban dengue eventually receded; but by 1953 another more virulent hazard, dengue hemorrhagic fever (DHF) was discovered in Manila, Philippines, and was vectored by *Ae. aegypti*. This DHF is even more deadly when accompanied by dengue shock syndrome, which is caused by multiple infections of different stains of dengue. Currently, dengue and DHF are major concerns in many parts of Southeast Asia; South America; Mexico; and the Caribbean, including Puerto Rico (Service 1989).

Yellow fever, a zoonotic disease of humans, is native to monkeys. Infected specimens of *Haemagogus* or *Aedes africanus* mosquitoes that vector the disease between monkeys occasionally bite and infect humans. Once the virus is in a human, *Ae. aegypti* effectively transfers the disease from person to person. Yellow fever vaccine has successfully reduced the risk of this disease (Service 1993).

The history of North America has seen epidemics of yellow fever and dengue fever, with humans as the principal host and *Ae. aegypti* the principal vector. For many years, these two deadly diseases have caused little or no concern in North America. However, concerns may rise with increased environmental issues surrounding the restoration of natural areas; and with the introduction and widespread colonization of anthropophilic species that can transmit these diseases (Reeves and Milby 1989, O'Meara et al. 1995).

Host Seeking

Host seeking in mosquitoes is an elaborate mixture of behaviors that can be influenced by many different situations. Mosquitoes are attracted by various signals that a typical host exhibits; and also by signals from inside the mosquito. Factors such as nutritional status, mating condition, and chemical or physical cues from the host, control whether a mosquito feeds or not (Takken 1996, Adams 1999). Moisture, carbon dioxide, heat, movement, and chemical signatures are some of the stimuli that attract mosquitoes to a host (Bar-Zeev et al. 1977).

After first emergence there is a lag period, usually lasting only 1 or 2 days, in which no host-seeking behavior is seen (Klowden 1994). The act of host seeking is induced in teneral mosquitoes by juvenile hormones and is inhibited by blood feeding. Abdominal distension arrests host-seeking behavior initially, with endocrine factors maintaining the behavior until after oviposition. Secretions from the male accessory glands also can affect host-seeking behavior, with nonmated females more likely than mated females to approach a host. Nutritional status of the female also can affect host-seeking behavior (Adams 1999). The initial activation to search for food (appetitive searching) at specific times of day is probably associated with hunger, circadian rhythms and possibly elevated atmospheric carbon dioxide (CO_2) levels. Flight becomes more focused in the final phase of host seeking when a mosquito happens upon the right conditions (attraction), such as a warm and moist flow of air (Lehane 1991). Activation and orientation is when the mosquito receives a signal that makes it change from internal to external impulses. These signals are very diverse and fluctuate in strength as the mosquito gets closer to the food source. She then must make a decision based on the presence of chemical signals (like CO_2 and other host odors) whether she wants to approach and feed on this potential host (Davis and Sokolove 1976). This type of breakdown of host seeking shows that it is a combination of events and not just incidental feeding (Lehane 1991).

Odor or chemical stimuli are usually the initial attractant that alerts mosquitoes of the presence of a potential host. Particular odors, such as body odor (or rather certain aspects of body odor) in combination with CO_2, increase host attractiveness to mosquitoes (Knols 1996).

Mosquitoes usually orient into a current of air because of an attractive odor trail. The odor might be related to breeding, feeding, or laying eggs. Several studies have identified some of the chemicals implicated in host seeking and location for several families of Diptera. For instance, the malaria vector (*Anopheles gambiae* s.s.) is attracted to some fatty acids similar to those secreted through human skin. Carbon dioxide is another compound that is a kairomone (or stimulator) for many species of mosquito (Daykin et al. 1965).

Most research aims to identify attractants for mosquitoes; but these studies may reveal a side effect leading to the identification of natural compounds that are repellent (allomones) (Takken 1996). For instance, even though CO_2 is a known attractant, increased levels of it can cause mosquitoes to leave an "at rest" position (Sherwood 1997). However, the levels of CO_2 do not seem to affect the landing rate of mosquitoes on hosts that have been treated with repellents (Carlson et al. 1992). This caused some researchers to believe that some chemicals may vary in their rate of attraction depending on the rate of circulation (Davis and Sokolove 1976).

Artificial Feeding and Host Models

To study blood-feeding insects and the diseases they carry we must rear the insects in colony and must entice them to feed in order to gauge reaction to specific treatments. Diseases transmitted by blood-feeding insects are the main reason for studying artificial feeding and host models. In the dark ages of science, scientists used volunteers for disease-transmission studies. For instance, Patrick Manson allowed mosquitoes that had fed on a malaria patient to infect volunteers who were not sick. This was the first recorded mosquito transmission of malaria in 1900 (Phillip and Rozeboom 1973). This kind of research has not been done, without severe consequences, in recent times. Disease-transmission studies must be carried out by alternative methods. First, the insect must be reared in colony, preferably by an artificial feeding method. Then an artificial host must be used to convincingly attract the mosquitoes during a test situation.

Most blood-feeding insects will not feed on blood or artificial diets that are free form. Feeding rituals are important (for example, probing or piercing the skin). A suitable situation will allow the insect to simulate natural conditions, with "skin" to pierce. If a membrane is provided with the diet, normal feeding is more likely to occur. The most suitable membrane must be chosen for the species being studied (Friend and Smith 1987).

Many types of membrane-feeding systems have been used either to rear blood-feeding insects; or for transmission studies (from the use of rat skin membranes to feed tsetse flies in 1912 to the use of guinea pig skin for colony maintenance in the 1950s). In the 1960s, surgical gauze and fibrin cloth, as well as a terylene mesh with agar were used. More recently, parafilm "M" and Baudrauche membranes from bovine intestines have proven successful (Galun and Margalit 1970). Butler et al. (1984), found that a combination of silicone and a fine cloth mesh was an effective artificial membrane for the feeding of stable flies, ticks and mosquitoes.

Feeding behaviors have been studied and certain factors have been determined that affect all stages of feeding (including probing, engorgement, and even egg production). Temperature, CO_2 levels, and presence and concentration of host odors can affect feeding behaviors (Bar-Zeev et al. 1977, Schreck et al. 1990). Optimum feeding temperature is between 28 and 40° C for most species. *Aedes aegypti* feed faster at 36°C or higher (Cosgrove and Wood 1995).

Blood feeding or hematophagy (probably evolved from sap-sucking ancestors) is present is several distinctly different groups of arthropods. Through the evolutionary process, almost all groups developed the same stimulatory tastes. Some components of blood are consistently phagostimulants for many groups of blood feeders. Mosquitoes feed on water, sugar water, or blood that is taken in relatively large amounts into the midgut. Blood feeding is instigated first by the correct temperature; and then by phagostimulants (such as adenine nucleotides) that are present in the blood. When adenine nucleotides are added, they can stimulate most Diptera to feed on pure saline solution (Galun et al. 1985, Galun 1987). High concentrations of platelets in solution can cause engorgement behavior by some blood-feeding insects (Galun et al. 1993). *Aedes aegypti* normally require a blood meal to develop a viable batch of eggs. However, it has been shown that the components of blood can be created with stock solutions and allow for a viable egg batch (Kogan 1990).

Galun stated that chemical activation of feeding is an adaptation to the presence of adenine nucleotide compounds found in blood. However, the attractant fractions are locked within the blood cells; and thus are not available to insect chemoreceptors. Insects are not attracted to blood by chemoreception. If attraction to blood is not chemoreceptive, then host attraction is not based on blood, but on outside factors (Galun et al. 1985).

Host Preference

In a general sense, host preference "denotes the species of host animal or animals from which a blood sucking insect obtains its blood meals," but host preference can be more specific than just identifying the species of host or position on a host (Lehane 1991, p. 14). Insects often appear to select specific individuals within a species. Vector species choose a host based on preferences that are controlled by stimulation from outside the vector. For instance, certain mosquitoes are zoophilic (animal seeking), others are anthropophilic (human seeking), and still others are ornithophilic (bird seeking). Within each host species, mosquitoes will choose individuals due to a diverse combination of selection pressures such as race, sex, age, host defensive behavior, use of bed nets, and ease of access from breeding sites (Gilbert et al. 1966, Burkot 1988).

Vertebrates have a specific pattern of body odor that is a mixture of sweat, sebaceous gland secretions, and bacterial by-products. Each vertebrate species is composed of specific types and numbers of glands (and their microflora) that create the recipe for that species' olfactory signal. For example, humans have a much higher density of eccrine sweat glands than any other vertebrate species. Eccrine sweat contains large amounts of L-lactic acid (which, when tested in conjunction with other skin odors and CO_2, increases the catch of *Ae. aegypti*) (Dekker et al. 2002).

Human studies have been done to determine variations in levels of mosquito attraction among certain populations. Attraction is quantified by counting actual bites received by human subjects; or by counting the probing attempts of mosquitoes when exposed to a human forearm. The perception of the general public is that certain individuals are more susceptible than others to mosquito bites. Studies have attempted to prove or disprove this assumption. Age and gender reportedly affect people's attractiveness to mosquitoes (Dekker et al. 2002). Khan et al. (1965) reported that Rahm used an olfactometer to record gender differences. He found that males were more attractive than females to *Ae. aegypti*. He also determined that babies were less likely than adult males to be bitten. However, he found no differences among humans within the same age group (Khan et al. 1965). However, in a more recent study, variation among men of approximately the same age was recorded. Individuals were assessed for long-range attraction and for close range or bite preference. In testing (which spanned more than 2 years) it was found that some individuals attracted more mosquitoes to their bed-nets than others did; and some individuals were fed on more than others were. Results indicated that mosquito preference for certain individuals does exist and remained consistent over a period of more than 2 years. However, long-range attraction was not consistent with bite preference. The individual who attracted the most mosquitoes did not receive the most bites. Perhaps different attractants are required for each situation; or more likely, perhaps some people respond more defensively to being bitten (Lindsay et al. 1993).

Charlwood et al. (1995) challenged the report produced by Alekseev stating that increased attraction is due to a pheromone released by already feeding mosquitoes. Results of Alekseev's study could not be reproduced with available species of mosquitoes (Charlwood et al. 1995). Many stimuli attract mosquitoes to a host, but most variation stems from the chemical composition of the host's skin secretions (Parker 1948). With anthropophilic mosquitoes, many host attractant simulations can be run with various stimuli that attract mosquitoes, but none seem to work as well as the actual human (Bar-Zeev et al. 1977). Studies in an olfactometer have shown that chemicals produced by humans (not including CO_2 and H_2O) dominate attraction of female mosquitoes from distances of 1 cm to 1 m away (Carlson 1979).

Some researchers believe that host preference is not a case of one particular chemical compound, but rather the presence of varying amounts or blends of compounds that release particular odors (McIver 1968). Mosquitoes probably recognize a host from a mixture of odors; and not just a single odor. This is because an appropriate host would be more easily chosen from a group of peers because of differences in the components (or levels of each component) of a mixture of odors; rather than try to distinguish differences in only one odor (Geier et al. 1996). It is not actual blood that attracts mosquitoes; instead, they respond to signals such as visual cues, heat, and chemical emanations that come from hosts containing blood (Klowden 1995). Combinations of odor and convection currents rising from the body are commonly thought to be the first signals from a human host that evoke responses in female *Aedes aegypti* (Eiras and Jepson 1994).

The importance of host preference of various species of mosquito is long recognized in the role of these vectors in transmitting diseases; however, it has been poorly explained (Reeves 1953). Preferential biting of some people more than others over time is important to epidemiologic studies, because it proves that, within a population, some people are at greater risk of mosquito borne disease than others (Lindsay et al. 1993).

Olfaction

Introduction

About one million species of insects have been identified, but only a few are known to vector diseases. This is probably because such a complicated evolutionary process (physically and behaviorally) is needed to transmit diseases. An important behavior is the ability to seek out a host. This involves steps that are triggered by some sort of signal that is received from a host. These signals start out as long-range attractants or activators; the most important of which are chemical cues received from the host (Klowden 1996). The ability of biting Diptera to locate a host requires a series of distinct behavioral responses. Olfaction (detection of airborne gases) plays an important part in the host-seeking behavior of blood-sucking arthropods; and is often used along with the other senses (Nicolas and Sillians 1989). First, mosquitoes use olfactory senses to travel great distances upwind while detecting a host. Blood-sucking flies use host odors as an important cue in host location (Alzogaray and Carlson 2000). Some of the clues mosquitoes use for long-range attraction are CO_2, sweat, and air-borne by-products of a foot bacterium (*Brevibacterium epidermis*). Once closer to a host, the mosquito can also use L-lactic acid, host temperature, relative humidity coming from the host, and maybe even some visual cues. More mosquitoes were collected from the bed-nets of pregnant women than non-pregnant women. This could be due to higher temperatures, larger body mass (Port et al. 1980), and possibly a larger amount of skin bacteria (Ansell et al. 2002).

Visual and physical cues are believed to lead to basic host location, with olfactory cues leading to the selection of specific hosts (Cork 1996). Odors are very important in controlling the behavior of insects. For instance, sex pheromones attract insects to potential mates. Chemicals from plants and flowers, or chemicals produced by a host species, can lead insects to preferred oviposition areas or to food. In female mosquitoes, odors also can be related to host seeking. Olfactory signals lead both the male and female mosquito to nectar for an energy source (Anton 1996, Burkett 1998).

To be able to detect food by its smell is complicated with many issues. First, there has to be a way of sorting through the many thousands of different chemical signatures floating through the air, even in tiny concentrations. Second, blends of odors can be more important as a food source or predator, than individual odors. And then, after an odor has been labeled as either predator or prey, the appropriate response must be activated, i.e. flee or feed. In other words, an organism has to correctly make split second decisions based on smell or risk being eaten. These decisions are not made easier by the fact that the odors are carried on turbulent plumes that can be interspersed with other stimuli. The organism must take into consideration the frequency of contact with an odor to correctly judge the distance to the source. The more often an odor is encountered the closer it is (Lemon and Getz 1999).

Evaporation and wind moving the volatile components in a "plume" structure (Murlis 1986) produce odor signals. These odor signals are translated by olfactory sensilla located on the antennae and the palpi. These olfactory sensilla have many pores in the wall of the sensilla where odor molecules are thought to enter. *Aedes aegypti* have sensilla trichodea of various sizes and shapes, some of which are shaped like grooved pegs and are less than 10 μm long. These peg-like sensilla, which can comprise up to 90% of the antennal sensilla in mosquitoes, are responsible for detecting chemicals related to oviposition sites, energy reserves (sugar sources), and some repellents. Mosquitoes also have olfactory sensilla called capitate pegs that are located on the maxillary palps, which serve in host location (Steinbrecht 1996).

Olfactory neurons project from the antenna to glomeruli in the antennal lobes. Within the glomeruli the olfactory neurons synapse with inter-neurons, some of which project to other regions of the brain, including the corpora pedunculata in the protocerebrum. Integration of the incoming olfactory signals leading to odor discrimination may occur at several levels but the corpora pedunculata in the protocerebrum are major integrative centers and probable sites of final discrimination (Mustaparta 1996). The way insects perceive chemicals has been split into two categories; chemoreception and olfaction. The categories are based on the type of stimuli or volatility of the chemical and also on the method of chemical transport (liquid versus vapor). They are also based on the morphology of the actual sensilla.

Contact chemoreceptive sensilla can have distinct outer shapes, with common attributes, such as the tip of the sensillum having only a single pore with unbranched dendrites of approximately two to ten cells that stretch all the way to the tip. In association with the chemoreceptive cell is often a mechanoreceptive cell which oversees movement of the sensillum. Stimulating substances are sent to the dendrites via the pore at the tip of the sensilla. The substance travels to the dendrite through a thick liquid that may even cover the tip itself. The type of liquid found in the sensilla as well as the rate of opening and closing of the pore itself may determine the actual rate of diffusion of substances across the dendritic membranes.

The presence of both sugar and amino acid receptors indicate the role of nutrients in inducing feeding. Lipids are important in both host plant selection and nutrition, but as of yet these receptors have not been identified. Behavioral studies have shown how important many chemicals are for host location, but the chemoreceptors for host selection have not been studied. Galun (1976) believed that olfaction was much more important for host selection than chemoreception (Städler 1984).

Mosquito Attractants

The primary mosquito species used in olfaction research is *Ae. aegypti* because it is an important vector that is easy to keep in colony and tends to have a short response time when exposed to stimuli. This ease of rearing and the importance of this species has established it as the leading laboratory animal in Medical Entomology (Geier et al. 1996).

Effective attractants used in conjunction with traps can be essential to the detection and possible control of infestations of *Ae. aegypti* and other insects. Several attractants have been identified for this particular species, but more are needed. Since many studies have identified sweat as an attractant, scientists have tried to emulate sweat or test individual components of sweat. The results have been mixed. Carlson et al (1973) reports that Roessler (1961) used an "artificial sweat" mixture of aliphatic acids and amino acids, that reportedly had a slight attraction, but noted that without the presence of CO_2 it was not effective. In 1968, Acree et al. isolated L-lactic acid from skin washes and found it, plus CO_2, to be attractive to mosquitoes. Carlson et al. (1973) reported that two – five carbon carboxylic acids with an α-substituent were good attractants for *Ae. aegypti*. Since esters were not found to be attractive, it can be inferred that a free carboxyl group is necessary in this equation (Carlson et al. 1973).

Many studies have tried to define the role of certain stimulants in the host-seeking behavior of mosquitoes (Dogan and Rossignol 1999). Other studies have shown that host odor plays a part in choice of host. Animals can attract insects from quite a distance, just by their odor. The actual distance has been calculated for several types of blood-sucking insects, and for mosquitoes it has been determined that a calf can attract mosquitoes from a distance of 15 to 80 m (Gillies and Wilkes 1969, 1970, 1972).

Many of the chemicals involved in olfaction have been identified, including CO_2, lactic acid, acetone, butanone, and octenol (Kline et al. 1990). *Aedes aegypti* have also been shown to be attractive to some estrogens in minute amounts (Brown 1966).

Almost all blood-sucking insects use CO_2 to assist in activating and orienting them for host location, and it is possible that some insects that are not blood feeders use it for the same purpose. It has been shown that CO_2 can act to stimulate a mosquito to focus on a particular host (Omer and Gillies 1971, Warnes and Finlayson 1985). It is usually found in the atmosphere at around 0.03% to 0.05%, with the occasional peak around 0.1% occurring at night in thick forests. Flying insects do not respond necessarily to the presence of CO_2 as much as to the concentrations, that are present. Mosquitoes can determine very small changes in concentration, as small as 0.05% in some tests (Mayer and James 1969), and other tests show that the changes can be as small as + 0.01% (Kellogg 1970). Carbon dioxide is also secreted in the sweat of hosts but most of a host's total CO_2 produced, approximately 4.5%, come from breathing (Eiras and Jepson 1991, Costantini et al. 1996, Nicolas and Sillians 1989, Sherwood 1997).

Carbon dioxide is important in host attraction, but not necessarily key to discrimination between species. Carbon dioxide indicates that a host is near, but other odors signify the species present. *An. gambiae* Giles *sensu stricto* and *Cu. quinquefasciatus* Say were able to distinguish between a calf and a human even though they were approximately the same weight and put off about the same amount of CO_2. Studies such as this indicate that CO_2 is not the key to host preference, but that there is an unknown scent component that allows anthropophilic mosquito species to select people over others (Dekker et al. 2002).

Normally, CO_2 is just one of many signals that are given off by the host. Carbon dioxide is one of those chemicals that works well with others. It can work in combination with one or more other host chemicals to create a different response than any single one would have elicited alone (Gillies 1980). Combinations of chemicals such as those found in human sweat have been shown to attract most species of mosquito. Lactic acid is a volatile chemical made during anaerobic metabolism that is found in many different bodily secretions of mosquito hosts (Davis 1988).

Lactic acid does not cause much excitement for insects unless it is combined with CO_2, then it becomes excellent at both activation and orientation (Smith et al. 1970, Price et al. 1979). Behavioral studies with *Ae. aegypti* and human sweat extracts have shown that L (+) lactic acid, along with many other compounds of human sweat, attract mosquitoes as long as they are in the presence of CO_2 (Acree et al. 1968, Davis and Sokolove 1976, Bowen et al. 1994), yet the human hand (or the whole entity) is much more attractive than any of the specific chemicals which have been tested (Cork 1996). It has also been shown that L-lactic acid by itself does not increase activation or attraction for *An. gambiae*, but when combined with a human hand or homogenous plumes of CO_2, it increases the response to the other factors (Dekker et al. 2002). When tested, in both skin rubbings and skin headspace, humans can differ by a factor of five in levels of L-lactic acid collected. This could be due to increased density of eccrine sweat glands, activity of the sweat glands or differing pH of the skin. For example, one study found that by adding 2-6 times the amount of L-lactic acid to one finger a shift in attractiveness could be accomplished. This amount of L-lactic acid is within the order of magnitude of naturally occurring differences in people. Which could indicate a contribution by L-lactic acid to intraspecific selection by anthropophilic mosquitoes (Dekker et al. 2002).

Host odor and host waste odor have been found to be highly attractive to tsetse flies. The identification of components of these odors led to the creation of baits for trapping tsetse flies. For example, 1-octen-3-ol was isolated from cattle breath and found to be an excellent attractant for the tsetse fly (Butler and Okine 1995). It also is an attractant that works on mosquitoes in the field as well as in the laboratory (Takken and Kline 1989), but only if it is combined with CO_2. A very similar component

(1-octen-4-ol) is a proven repellent. This ox breath compound (1-octen-3-ol) generally attracts mosquitoes that primarily feed on mammalian species. Octenol and acetone, both components of the breath of oxen, increase the catch in CO_2 baited traps set for tsetse flies (Hall et al. 1984, Vale and Hall 1985).

Several chemical signals together would seem to be a better indicator of the presence of a suitable host than a single signal. For example, CO_2 by itself only alerts the insect that there is a possible meal nearby, not that the meal is one that would be appropriate or normally chosen. A single chemical signature could be released from many inappropriate entities. However, a combination of chemical signals is less likely to be duplicated in the same pattern of chemical proportions, therefore, by responding to combinations of signals, an insect can greatly reduce the amount of time and energy spent searching for food. Since insects show preferences within a host species, the ability to detect chemicals from a distance could allow for selection of a host that is not nearby. Odors created by a mixture of body chemicals may be specific for a species or even an individual. This is specifically true for ticks finding hosts in response to CO_2 and tick pheromone (Norval et al. 1989). In most cases the combination of chemical signals which attracts an insect to a host is probably just a proportional map of non-specific chemicals which identify an overall group. It would make sense that these chemical "maps" would also allow for avoidance of a host or group of hosts which is not particularly appetizing to the insect in question (Lehane 1991).

In female *Ae. aegypti*, cells on the antenna, specifically "chemoreceptor neurons on the grooved-peg (A3) sensilla" are used to detect attractant odors such as L-lactic acid and certain carboxylic acids as well as other body odors. They also have cells on the maxillary palps that are used to detect CO_2 (Davis and Sokolove 1976). The presence of CO_2 is thought to act, not only as a long-range attractant, but also as an activator for host seeking. Other chemicals have been identified as attractants, but not as a stimulant for blood feeding. For instance, certain phenols and indoles appear to evoke an oviposition response from mosquitoes.

Host Odors

It is suspected that human emanations other than CO_2, H_2O and lactic acid act to attract mosquitoes. For example, residues left on glass surfaces are attractive to mosquitoes, even when no CO_2 is added (Carlson et al. 1992, Bernier et al. 1999, Bernier et al. 2000, Bernier et al. 2002). Behavioral studies on *Aedes* with human odors have shown that there are still compounds of host odor which are attractants and which have not yet been identified (Pappenberger et al. 1996). One school of thought believes that a human hand is much more attractive than the known components (which are simulated in the lab) because of the quality of the chemicals or "signals" that are coming from the hand, and not necessarily because something has not been identified (Khan et al. 1967). However, more recent research has isolated more than 275 compounds that have been identified as mosquito attractants (Bernier et al. 2000).

Anopheles gambiae Giles *sensu stricto* is a nocturnal anthropophilic species that relies primarily on olfaction or kairomones to locate a host (Takken 1991, Knols 1996). Many researchers have attempted to identify these host kairomones without success. Carbon dioxide is important for some species but not for others, and the same is true of lactic acid. Human sweat samples have also been identified as attractant for *Ae. aegypti* (Skinner et al. 1965, Eiras and Jepson 1991, 1994) and for *An. gambiae* (Meijerink et al. 2000) although it could not be replicated with fresh sweat. It was found however, that incubated sweat did become attractive to *Anopheles*. Incubated sweat is very different than fresh sweat, in that it is no longer acidic (lactic acid) but alkaline because of by-products of urea digestion by bacteria. There is an indication that skin flora could be the major component of kairomone production of human mosquito attractants (Braks and Takken 1999).

Determination of Attractants

Repellent action, or repellency, is defined as "a substance that caused orientation away from its source" (Dogan and Rossignol 1999). An attractant is a cue that causes an organism to make directed movements toward it and a stimulant is a cue that causes an increase in the occurrence of a specific behavior (Collins and Blackwell 2002).

The most common method of identification of repellent or attractant chemicals is a "trial and error" method. This method employs the strategy where products are put into traps or baits or on skin to see if they work. The next most common method is to "ask the insect to choose its preference". Insects and chemicals are placed in a test chamber and the insect's reactions are recorded. In 1963, Dethier developed an olfactometer that utilized behavioral choices, so that numbers could be attained for preference studies. The optimum systems have multiple choices with all aspects held the same for each choice except for the test component. A third approach is to monitor the insect's neural pathways while presenting the insects sensors with test chemicals. Probes placed in the olfactory lobe of the insect brain can measure the activity of the insect's olfactory sensilla, recording the response of an insect to a specific odor (Butler and Okine 1995).

For instance DEET, a well-known repellent, is actually considered an inhibitor. It works by inhibiting the mosquito's ability to sense L-lactic acid, effectively blocking antennal receptors. However, there are studies that suggest that at some levels DEET appears to act as an attractant instead of an inhibitor (Hoffman and Miller 2002).

Summary

Aedes aegypti became one of the most common species of vector mosquito in tropical regions by the 1700s and has been living with people much longer than any other vector species recorded (Laird 1989). Even though there are practices that could be incorporated to decrease the risk of disease transmission by *Ae. aegypti*, it still remains a primary disease vector in some countries.

A mosquito's search for blood is a necessary activity to ensure survival of the species. Internal clocks and hormones as well as external stimuli draw mosquitoes to a typical host. Host seeking for blood feeders appears to be primarily directed by odors. One might think that odors would be more important to insects that have restricted visual contact with hosts, but studies have suggested that it is actually more important in species that live in relatively open areas (Lehane 1991). *Aedes aegypti* first identify a host through vision. During host seeking, *Ae. aegypti* are affected visually by dark colored surfaces and are sensitive to moisture (Browne and Bennett 1981). However, this first visual signal could be very confusing if it were the only one available. There are many objects other than humans, such as rocks, lone trees and many man-made objects that might attract *Ae. aegypti* from a distance (Bowen 1996). Host kairomones and, in some cases, mosquito pheromones act as chemical stimulators for mosquitoes (Ahmadi and McClelland 1985). These kairomones that are produced by a host, inform mosquitoes that the object they have visually identified is not an inanimate object.

It is not actual blood that attracts mosquitoes; they, instead, respond to signals such as visual cues, heat and chemical emanations that come from hosts containing blood (Klowden 1995). Combinations of odor and convection currents rising from the body are commonly thought to be the signals from a human host that evoke responses in female *Ae. aegypti* (Eiras and Jepson 1994). However, after initial attraction it has been shown that components of blood are necessary to stimulate feeding (Galun et al. 1985). This is especially true when researching disease transmission or any other study that requires the use of an artificial host instead of human use.

The study of olfaction is important to understanding the possibilities of mosquito control (Anton 1996). Olfactory regulated host seeking behavior in mosquitoes is acknowledged by most people in the field of mosquito research, even though a lot is yet to be understood. Many studies on mosquito olfaction with many species of mosquito have come to the same conclusion, which is that host interaction is directed by olfactory signals.

Odor signals are created by a host and are detected by olfactory neurons in the mosquito's antenna, these neurons alert the protocerebrum that a possible blood meal is near because of the distinct pattern of specific chemicals that have just been registered. These chemicals have different values at certain stages of host seeking. Tests show that one olfactory cue, CO_2, although crucial in the first stages of host seeking, is not as important in the following stages. Other chemicals come into play as the mosquito gets closer to the host (Healy and Copland 1995). An actual organism can draw mosquitoes from a greater distance than an artificial source of CO_2, thus proving that something more than CO_2 is involved in attraction (Nicolas and Sillians 1989). This also implies that some chemical or chemicals should be considered orientation components of attraction (Kennedy 1978). Individual host odors determine how a mosquito will respond to CO_2. This response can change depending on the species of mosquito (Costantini et al. 1996).

There are three main groups of body "emanations": skin secretion, breath, and urine. Host attraction is probably caused by a combination of these groups. The way the different components and chemicals from each group work together is probably very important in determining an individual's level of attraction (Takken 1991).

The need for more studies is evident in the ongoing attempts to control mosquitoes and the diseases they carry. Many areas of science are devoted to improving the quality of life throughout the world by reducing the risk of disease transmission. The goal of this type of research is to disrupt the balance between host and vector. The best way to control a vector borne disease is to disturb the frequency of contact between the two. Old methods of control have been shown not to have a lasting effect on dissipating mosquito populations, and so, new methods are being developed. If contact between the host and vector is decreased, the incidence of disease also would decrease (Takken 1996).

This kind of control is being explored with the use of new commercial traps that are currently being marketed for the consumer.

CHAPTER 2
COMPARISON OF STANDARD ARTIFICIAL HOST MEDIA COMPONENTS

Introduction

The testing of mosquito attractants and repellents as well as transmission studies on diseases have long employed artificial medium within an artificial host. This is advantageous in that there is no exposure of live hosts to disease nor is there host immune response interference with feeding (Butler et al. 1984).

Many factors affect the feeding behavior of mosquitoes. For an artificial medium to be effective it has to adhere to basic standards, induce a female mosquito to investigate, probe and try to feed (Friend and Smith 1987). Temperature requirements are essential, and while CO_2 is not strictly necessary for attraction, it can influence probing and feeding behavior (Bar-Zeev et al. 1977, Schreck et al. 1990, Cosgrove and Wood 1995). Carbon dioxide also can act as an activator that influences blood-feeding insects to focus on a particular host (Omer and Gillies 1971, Warnes and Finlayson 1985, Dekker et al. 2002). Some compounds that have previously been identified as attractants do not work as well without the presence of CO_2 (Acree et al. 1968, Carlson et al. 1973, Davis and Sokolove 1976, Bowen et al. 1994).

Another factor in an effective artificial host system is the membrane. Membrane feeding techniques have been used in studies dealing with the transmission of pathogens by different hematophagous arthropods. Membrane feeding is also helpful when looking at behavioral or nutritional studies for sucking insects. A membrane has to successfully mimic the skin of the natural host in order to induce probing. If this is not accomplished, results may be skewed. Low feeding attempts could be the result of an undesirable membrane, not an undesirable test compound (Friend and Smith 1987).

Blood has been shown to be a stimulant for mosquito feeding, however, the stimulatory fraction of blood that is confined within the red blood cells is adenine nucleotides (Galun and Margalit 1970). Although blood is not an activator for probing, once probed it can be an activator for sustained feeding (Galun et al. 1993).

The objective of this study was to compare four formulations of artificial host media used in attraction studies, to determine if it was possible that one or more components of the artificial media were masking the compound being evaluated.

Methods and Materials

Mosquitoes Used in Trial

Aedes aegypti, raised in colony at the University of Florida Medical and Veterinary Entomology Lab, were used in this research (Gerberg et al. 1994, McKenzie 2000) (Figure 2-1). Larvae were raised on a ground fish food diet and adults were fed sugar water until used in trials. Trials in this study used 160 adult, 3-11- day-old, nulliparous, female mosquitoes. Mosquitoes attracted to a human hand held outside the cage screen were aspirated into five Tygon® tubes (St. Gobain Performance Plastics, Akron, OH) (Inside Diameter: ¼") (Figure 2-2) by the primary researcher. The female mosquitoes were counted and the males were removed. Mosquitoes were not left in the tubing for more than 10 minutes before release into test chamber of the olfactometer. The mosquitoes were placed in the olfactometer by gently squeezing air through a squeeze bulb that fit on the end of the Tygon® tube through five alternating ports in the Lexan® (GE, Pittsfield, MA) top cover, 32 mosquitoes in each tube, to decrease positioning effects. Olfactometer recording was begun within minutes of mosquito release.

Most olfactometer systems require a preconditioning period, where the mosquitoes are released into the system for some predetermined time to allow for acclimation to the new environment. The olfactometer used in this research does not require a preconditioning period. This step is eliminated because of the extended trial length (as compared to most cage type olfactometers).

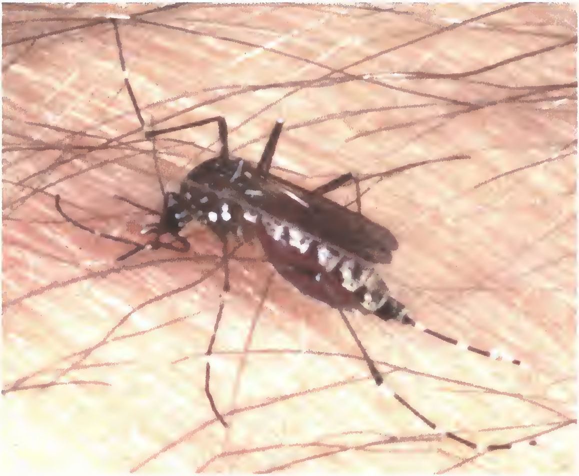


Figure 2-1. *Aedes aegypti* feeding on human arm

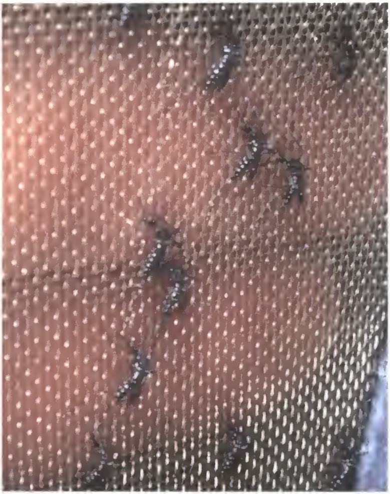


Figure 2-2. Female *Aedes aegypti* coming to a hand held outside a rearing cage. This method is used to collect (for use in attraction trials) mosquitoes that are interested in actively blood feeding. Mosquitoes were not allowed to feed on blood nor were they allowed to contact human skin prior to this testing.

Colony

The colony maintenance schedule was set up in a 7-day cycle based on a modification of schedule by Gerberg et al. (1994) (McKenzie 2000).

- **Step 1 (Day 1):** Four or five (2" x 3") strips of previously collected (from Medical and Veterinary Entomology established colony) egg-covered blotter paper were placed in tap water with a small amount of ground fish food. The eggs were then incubated at room temperature.

Step 2 (Day 2): Twenty-four hours after step 1, forty-eight (14" x 9") enameled baking pans were filled halfway with 1250 mL of deionized water. Two mL (0.74 g) of Tetramin® (Tetra Werke, Melle, Germany) ground fish food was then placed in each pan along with 2 drops of first instar larvae, each drop containing approximately 125 larvae. The pans were kept in Hotpack® (Hotpack, Philadelphia, PA) Temperature Humidity Chambers. Chamber temperature was kept at 32° C with the humidity at 85%.

- **Step 3 (Day 5):** Feed 2nd and 3rd instars. Added 1 mL (0.37 g) of ground fish food to each pan.
- **Step 4 (Day 7):** Collect pupae and 4th instars. Contents of a pan were poured through a filter with clean deionized water, and collected pupae were then placed in cups of deionized water and put inside screened colony cages (30 cm x 30 cm x 30 cm, produced by both AMBI and BioQuip) for emergence.

After emergence the mosquitoes were maintained on a 10% sucrose/water solution under a 14 hr:10 hr (Light:Dark) fluorescent lighting photoperiod, with room conditions at 28° C and 82% RH. One of the three fluorescent light bulbs was replaced with a plant grow light to more closely resemble sunlight.

Mosquitoes that were not used in testing were transferred to an egging cage where they were fed citrated bovine blood (3.17 g sodium citrate per liter bovine blood) and allowed to oviposit on blotter paper, which was removed weekly and dried to maintain a stock of eggs for weekly use.

Olfactometer

The olfactometer used in this research was designed by Dr. J. F. Butler and was supported by a grant from International Flavors and Fragrances, Inc. This olfactometer is an electronically monitored feeding detector that offers the simultaneous choice of 10 artificial hosts with associated air/treatment ports (Butler and Katz 1987, Marin et al. 1991, Wilson et al. 1991, Okine 1994, Butler and Okine 1995). The pie-type olfactometer contains ten air inlet ports around the perimeter of the device. Air treatment is introduced through a side port and is removed at the same rate from the center of the olfactometer with negative pressure, creating a continuous plume. Airflow rates for each host are approximately 0.6 mL/ min. Each port is located just underneath an artificial host/electronic feed monitoring device. The air supply is augmented with moist CO_2 that is introduced into the airstream by a computer controlled valve at a rate of 250 mL/ min (4 minutes on and 6 minutes off). This was used to expose treatments and mosquitoes to expected CO_2 near the artificial host, in case material being evaluated was affected by host CO_2 production. The olfactometer is housed in a temperature controlled, light-proof, Faraday-cage room (Lindgren Enclosures, Model No. 18-3/5-1) (Okine 1994, Butler and Okine 1995). (Figure 2-3 and Figure 2-4) (US Patent No. 5,134,892).

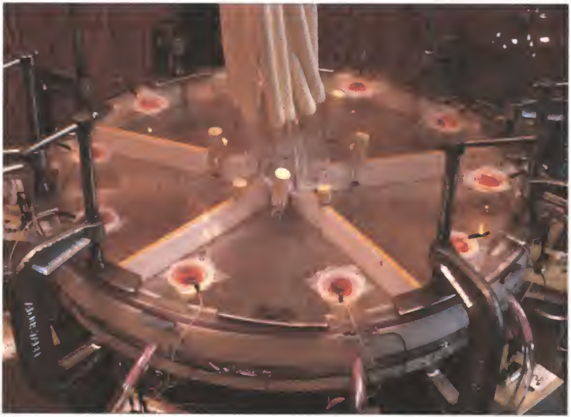


Figure 2-3. Ten port, pie-type olfactometer designed by J.F. Butler and grant supported by IFF, used in mosquito attraction trials at the University of Florida. Current view shows two-choice, five repetition set up.

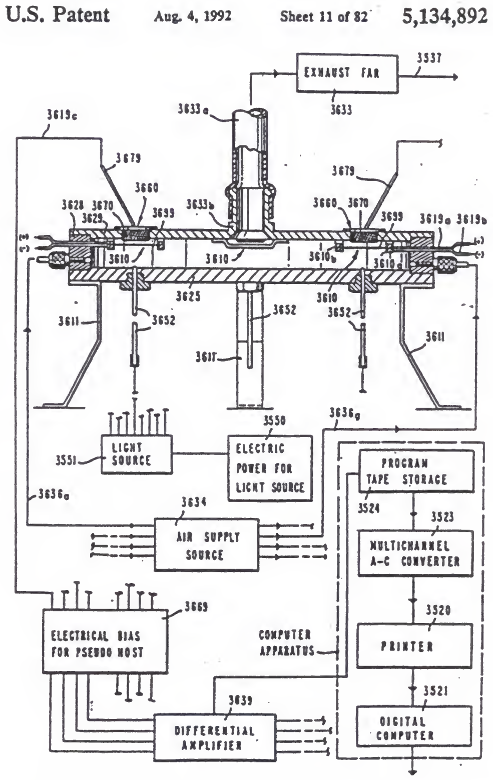


Figure 2-4. Schematic of UF/IFF olfactometer, Figure I-La, sheet 11 of US patent # 5,134,892, Aug.1992 (Wilson et al. 1992)

Artificial Host

The standard (3776) artificial host in the test chamber is an attractant food source consisting of a mixture of 33 mL of bovine blood (plus sodium citrate as an anticoagulant), 1.66 g of agar (U.S. Biochemical Corp, Cleveland OH), and 100 mL BSS ® Plus intraocular irrigating solution (Alcon Labs, Fort Worth, TX). BSS® Plus consists of two parts. Part I is a sterile solution containing "sodium chloride 0.395 mg, dibasic sodium phosphate 0.433 mg, sodium bicarbonate 2.19 mg, hydrochloric acid and/or sodium hydroxide (to adjust pH), in water for injection". This is a basic saline solution that is not attractive to blood-feeding insects (Galun and Margalit 1972). Part II is a sterile concentrated solution in which each mL contains the following ingredients: "calcium chloride dihydrate 3.85 mg, magnesium chloride hexahydrate 5 mg, dextrose 23 mg, glutathione disulfide (oxidized glutathione) 4.6 mg, hydrochloric acid and/or sodium hydroxide (to adjust pH), in water for injection". This solution has components that are known attractants to blood-feeding insects (Galun 1967, Galun et al. 1985A).

After Part I and Part II are combined each mL of BSS ® Plus solution contains: "sodium chloride 7.14 mg, potassium chloride 0.38 mg, calcium chloride dihydrate 0.154 mg, magnesium chloride hexahydrate 0.2 mg, dibasic sodium phosphate 0.42 mg, sodium bicarbonate 2.1 mg, dextrose 0.92 mg, glutathione disulfide (oxidized glutathione) 0.184 mg, hydrochloric acid and/or sodium hydroxide (to adjust pH), in water for injection" and has an approximate pH of 7.4 (Preceeding taken from product insert for BSS ® Plus Ocular Solution).

This recipe makes approximately 130 mL of artificial host medium, which could be used for about one week. The mixture was used as a formula that mosquitoes detect as a food source. Sensing blood as a known feeding stimulant and BSS® II with known attractants insects are drawn into questing and feeding behavior, however, with the addition of agar the mixture is not a liquid or easy to ingest (Galun and Margalit 1972). Since the mosquitoes are unable to feed to repletion on the food source, they continue to actively seek new hosts for the duration of the trial. For this study, three other formulations of media were also created (Table 2-1). The artificial host consists of a food source in a shallow well, actually the underside of a 35 mm plastic film canister lid, that is sealed with a 4 mm retaining ring cut from the top of the film canister and a silicone membrane used as an "artificial skin" (Butler et al. 1984, Burkett 1998). This results in a self-contained treatable unit, which is identical in all choices except for treatments to the surface of the membrane or the air-stream.

Table 2-1. Variations of artificial host media used in study

Number	Ingredients
3776	Bovine Blood, Agar, BSS I* and BSS II*
3777	Agar and BSS I*
3778	Agar, BSS I* and BSS II*
3779	Bovine Blood, Agar and BSS I*

* BSS I and BSS II are sold as an irrigating ocular solution by Alcon Labs.

In these trials, nine of the ten "host" membranes were treated with human secretions, while the tenth membrane served as a control. The well or "artificial host" is placed membrane side down into the mosquito chamber of the olfactometer (Figure 2-5 a & b). Air is injected in through ports located in the side plate of the olfactometer directly under each host and exhausted out through the center of the top cover plate of the olfactometer. Under each host is a fiber-optic white light source, which sets minimum light standards on all hosts. These are the only light source in the room. These are arranged by dual infrared (IR) sources at 940 nm at 150 hz, which impinge on the artificial host from the base-plate and present some expected host IR. Each host is illuminated identically, reducing variations in light intensity (Figure 2-6).

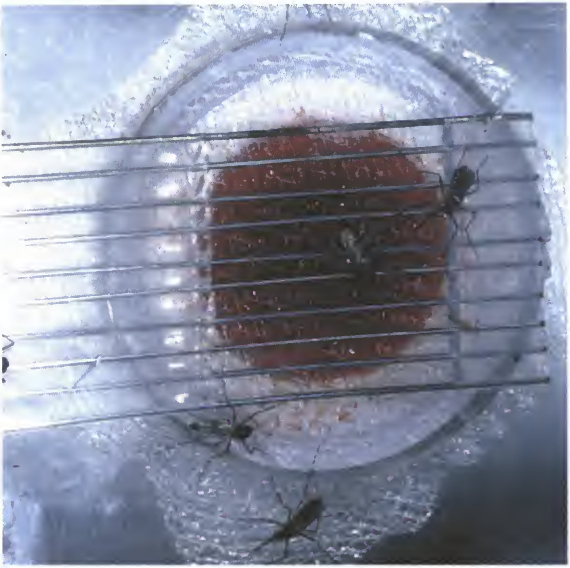


Figure 2-5a-b. *Aedes aegypti* investigating artificial host in the UF/IFF olfactometer. A charge (electrical signal) is produced by feeding. The signal is differentially amplified and data logged. A) Close-up of artificial host with mosquitoes on sensor. B) *Aedes aegypti* probing into the artificial host in the olfactometer test chamber.

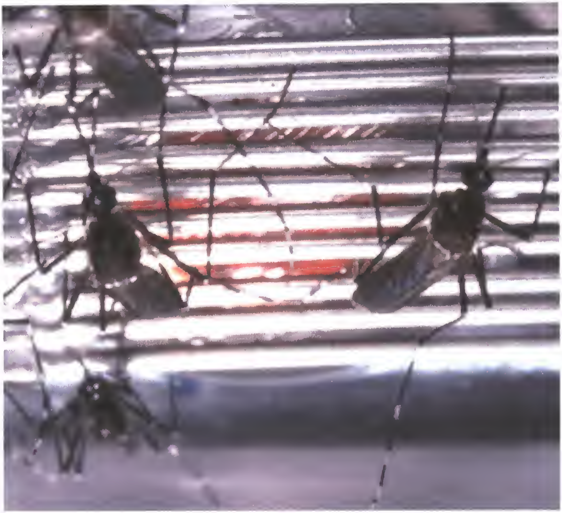


Figure 2-5 Continued

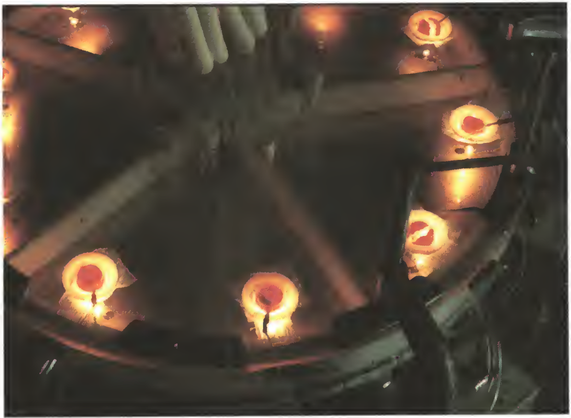
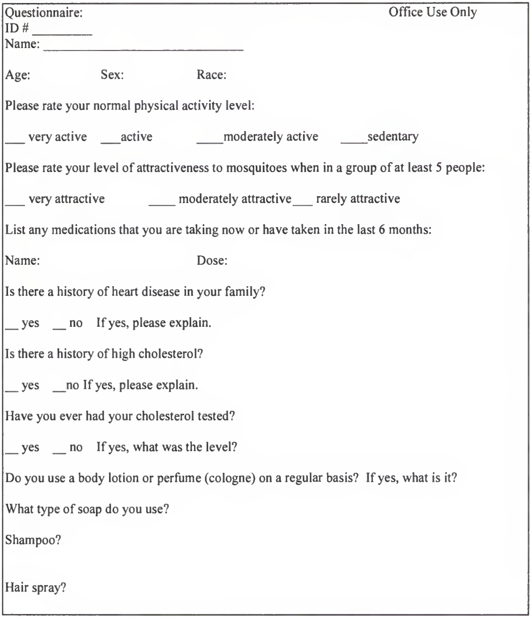


Figure 2-6. Working view of olfactometer showing artificial hosts, light arrangement and two choice configuration

Volunteer Information

The first step was to outline and identify the current study for the Institutional Review Board (IRB), which is the panel that regulates human use trials at the University of Florida. The IRB granted, under permit IRB # 210-1998, people to be asked to volunteer for this study if they had an opinion on their mosquito attraction level. Volunteers were asked to read and sign an informed consent form (Appendix A) and also fill out a contact questionnaire sheet (Figure 2-7), in which brief medical histories and current medications were noted for the duration of this trial. The subjects were asked to rate, from their experiences, their attractiveness to mosquitoes. The ratings ranged from rarely attractive to very attractive. Some of the subjects had participated in previous mosquito attraction trials, and these subjects based their ratings on the results of the previous trials. Self- ratings are listed in Table 2-2.



Questionnaire: Office Use Only
ID # ________
Name: ____________________________

Age: Sex: Race:

Please rate your normal physical activity level:

___ very active ___active ____moderately active ____sedentary

Please rate your level of attractiveness to mosquitoes when in a group of at least 5 people:

___ very attractive ____ moderately attractive___ rarely attractive

List any medications that you are taking now or have taken in the last 6 months:

Name: Dose:

Is there a history of heart disease in your family?

__ yes __ no If yes, please explain.

Is there a history of high cholesterol?

__ yes __no If yes, please explain.

Have you ever had your cholesterol tested?

__ yes __ no If yes, what was the level?

Do you use a body lotion or perfume (cologne) on a regular basis? If yes, what is it?

What type of soap do you use?

Shampoo?

Hair spray?

Figure 2-7. Volunteer information questionnaire completed by each subject prior to the beginning of the *Aedes aegypti* attraction studies.

Table 2-2. Self-rating of mosquito attractancy by trial subjects

Subject	Attractancy (Self rating)
8001	Very
8002	Rarely
8003	Rarely
8004	Very
8005	Very
8006	Very
8007	Moderately
8008	Rarely
8009	Rarely

Membrane Preparation

The silicone artificial skin (Butler et al. 1984) used in this study was used to collect secretions from nine subjects. Each subject was assigned a number from 8001-8009 and was given a 4" x 4" membrane to wear for four hours (Figure 2-8). The subjects were instructed to wear the membranes against the skin with complete contact for a four-hour period (Figure 2-9) and then to remove the exposed membranes, place them in a Teflon® (Dupont, Wilmington, DE) sealed glass vial and keep the vial with the membranes in a sealed, number identified baggy in the refrigerator until returned to the lab. The membrane and paraffin backing was assumed to absorb the secretions of the subject during the four hour period, trapping the odors and chemicals in the artificial skin until exposed in the olfactometer (Figure 2-10). The numbered membranes were cut and used for four trials. The sealed exposed membranes were usually from one to seven days old when evaluated. All aspects of the membrane preparation were handled carefully using latex or nitrile gloves to reduce odor contamination by technicians.

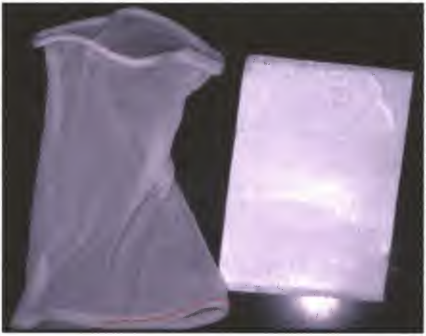


Figure 2-8. New membrane (on right) and nylon stocking (on left) were given to subjects to wear on their forearms.

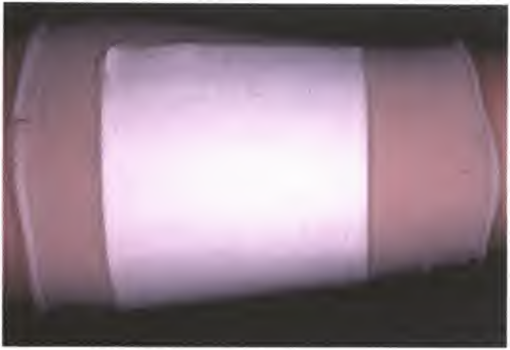


Figure 2-9. Demonstration of proper positioning of membrane flat against the skin of the forearm underneath the stocking, which holds the membrane snug against the subject's skin.

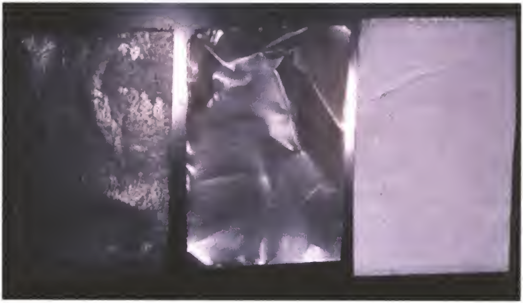


Figure 2-10. Membrane formation. The layer which goes against the skin (extreme left) is a very thin, 10-15μm minimum thickness "artificial skin," followed by a parafilm membrane (center) that isolates secretions on the membrane. On the right is a paper backing that helps to protect and keep the complex together (Butler and Okine 1995).

Data Recording

The artificial host was placed within the top of the olfactometer cover over a detector grid as a negative electrical contact. The detector grids consisted of a ten-strand wire reference sensor connected to one side of a differential amplifier (Figure 2-4) and were aligned from the outer perimeter of the olfactometer and below the "skin" of each artificial host. A positive contact probe was inserted into the top of the artificial host's blood agar and connected to the other side of the differential amplifier. The electrical connection from the artificial host and mosquito resting on the sensor were used to monitor mosquito activity. The inverted hosts in the olfactometer took advantage of the mosquito's preference (geotaxi) to feed in the inverted position in the chamber, thus increasing feeding contact. When a mosquito makes contact with the wire grid and touches or probes the agar, an electrical charge is produced, amplified, and recorded by the computer as bite-seconds. The differential amplifiers used were designed by J. Greenberg, Gainesville, Florida, and operate at 10,000x amplification (Butler and Okine 1995). A program called Medusa© collects, stores and datalogs input signals to individual host files on an Apple Power Macintosh 7100/80AV computer with Strawberry Tree™ hardware and software (produced by Strawberry Tree incorporated), at one and ten minute intervals. Bite-second data are cumulative for the eight hours of each repetition.

Procedure

This study consisted of four trials each consisting of ten eight-hour replications (individual tests that were run once or twice a day, during at least one prime feeding time, comparing subjects). Replications were used in the final statistical analysis if they had no mechanical, electrical or behavioral complications, such as amplifiers not communicating with the computer or sensors shorting, insects trapped behind sensors or no activity recorded for either standard controls 3776, 3777, 3778, or 3779 during a replication.

Volunteers were recruited by word of mouth for this trial. From a pool of approximately 60 volunteers, nine subjects were chosen based upon previous testing, personal opinion of mosquito bite frequency or medical histories. Determination of inclusion also was based on availability.

The volunteers were assigned an identification number and grouped with one of the untreated media compounds in the olfactometer for attraction rating. Ten repetitions were used for each trial and statistical analysis was run to determine significance. For each trial, subjects and a medium compound were randomly positioned within the olfactometer.

Trial 1. This trial tested standard 3776 (agar, blood, and BSS® Plus, Part I and Part II) versus nine human subjects, randomly positioned within the olfactometer. This trial was considered the model trial, as 3776 has been used in this lab for years as the standard artificial host medium. All results will be compared to this trial.

Trial 2. This trial tested 3777 (agar and BSS® Part I only) versus nine human subjects, randomly positioned within the olfactometer.

Trial 3. This trial tested 3778 (agar and BSS® Plus, Part I and Part II) versus nine human subjects, randomly positioned within the olfactometer.

Trial 4. This trial tested 3779 (agar, blood, and BSS® Part I only) versus nine human subjects, randomly positioned within the olfactometer.

Statistical Analysis

The trials were designed as randomized multi-choice tests, with 10 randomly placed artificial hosts. Medusa 2.1.2 F&B (Data logging of contact seconds of feeding and biting) software (designed by Nick Hostettler, Gainesville, Fl) was used to consolidate and analyze the number of bite-seconds per sample over an eight hour period. Normalized data were compared to the standard using a one-tailed *t*-test to determine whether there was any significant difference between samples. ANOVA was used to separate interaction as an independent error term to avoid misrepresenting actual significance. Data were normalized by a standardizing equation (Square root of n+1).

Results and Discussion

Trial 1

Ten repetitions were recorded for trial 1. Data were normalized using the equation: SQRT of n + 1 (Table 2-3). Attractiveness was measured in average bite-seconds per repetition. Figure 2-11 shows the normalized data graph with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 2-4). Therefore, even though each treatment is different it can be affected by the two treatments next to it. In this type of olfactory research, the interaction or "nearest neighbor" effect is combated with the use of randomization within each replication, multiple replications, normalization and a one-tailed *t*-test to compare treatments to each other (McKenzie 2000).

In this trial, one subject was significantly more attractive than 3776 (blood, agar and BSS$^{®}$ Plus) ($\alpha = 0.05, p = 0.04$, and one subject was significantly less attractive than 3776 ($\alpha = 0.05, p = 0.03$), (Table 2-5).

Table 2-3. Normalized bite-second data for human subjects in trial 1 of artificial media comparison study

Repetition	3776	8001	8002	8003	8004	8005	8006	8007	8008	8009
1	16.35	61.90	77.52	29.39	26.64	27.50	18.20	67.29	73.94	22.91
2	89.23	13.07	29.40	56.13	75.18	55.16	32.64	94.26	89.62	91.85
3	88.49	21.70	26.75	16.14	18.50	35.26	58.15	55.92	10.25	20.61
4	16.90	46.29	34.20	24.74	9.07	24.80	31.59	77.18	65.63	13.24
5	13.40	72.80	58.33	48.27	59.55	10.80	10.65	64.75	20.83	17.48
6	15.54	21.19	26.28	61.59	35.50	26.60	105.30	21.71	42.10	11.66
7	41.73	47.99	44.94	35.39	10.99	15.04	26.31	98.23	14.27	57.89
8	84.89	32.43	37.56	4.70	69.06	15.96	56.43	74.20	29.91	45.45
9	61.40	54.47	48.61	17.91	49.52	14.04	92.21	96.53	52.09	32.37
10	34.19	67.85	36.73	51.57	62.27	29.23	14.87	56.98	65.87	25.30

* Where 3776 contains blood, agar and BSS$^{®}$ Plus

Table 2-4. Analysis of variance for trial 1 of artificial media comparison study to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	8625.59	9	958.40	20.78	<0.0001	1.89
Treatment	12274.49	9	1363.83	29.57	<0.0001	1.89
Interaction	44083.48	81	544.24	11.80	<0.0001	1.29
Error	32280.63	700	46.12			
Total	97264.18	799				

Table 2-5. Standard two-sample *t*-test for trial 1 of artificial media comparison study, assuming unequal variances, comparing bite-second data for human subjects

	3776	8001	3776	8002	3776	8003	3776	8004	3776	8005
Mean	46.21	43.97	46.21	42.03	46.21	34.58	46.21	41.63	46.21	25.44
Variance	1033.05	439.71	1033.05	258.60	1033.05	367.21	1033.05	609.29	1033.05	171.36
P(T<=t) one-tail	0.43		0.36		0.17		0.36		**0.04**	

	3776	8006	3776	8007	3776	8008	3776	8009
Mean	46.21	44.64	46.21	70.70	46.21	46.45	46.21	33.88
Variance	1033.05	1072.71	1033.05	543.21	1033.05	743.51	1033.05	625.77
P(T<=t) one-tail	0.46		**0.03**		0.49		0.18	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.05$.

***Where 3776 contains blood, agar and BSS® Plus.

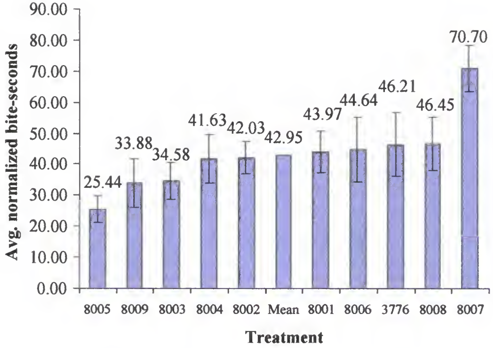


Figure 2-11. Normalized data graph for trial 1 of artificial media comparison study, showing the average bite-seconds (for ten repetitions) versus the overall mean, plus the standard error. Where 3776 contains blood, agar and BSS® Plus

This trial used the standard medium compound used in the UF, Medical and Veterinary Entomology, Research lab to determine attractant or repellent compounds. It is the base for chemical as well as human essence attraction trials (Butler personal communication). As has been shown in the past using this artificial medium, out of a group of nine subjects at least one will have something added to it that makes it significantly more attractive than the untreated membrane and at least one subject will have something added that makes it significantly less attractive than the untreated membrane (McKenzie 2000). There is a possibility that the additives are naturally occurring attractants found in the human skin or naturally occurring inhibitors that interact with the artificial medium to either enhance the phagostimulatory factors found in media or to hide them (Galun and Margalit 1970, Bernier and Posey 2001).

Trial 2

Ten repetitions were recorded for trial 2. Data were normalized using the equation: SQRT of n + 1 (Table 2-6). Attractiveness was measured in average bite-seconds per repetition. Figure 2-12 shows the normalized data graph with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 2-7).

In this trial, all subjects were significantly more attractive than 3777 (agar and BSS® Part I) ($\alpha = 0.10, p = 0.08, p = 0.09, p = 0.09, \alpha = 0.05, p = 0.02, p = 0.02, p = 0.05, \alpha = 0.005, p = 0.001, p = 0.004, \alpha = 0.0005, p = 0.0002$), (Table 2-8).

Table 2-6. Normalized bite-second data for human subjects in trial 2 of artificial media comparison study

Repetition	3777	8001	8002	8003	8004	8005	8006	8007	8008	8009
1	26.19	9.74	31.81	66.88	90.31	15.35	59.31	40.11	78.86	33.39
2	18.57	23.08	44.56	66.01	52.25	21.85	2.70	8.66	55.67	27.30
3	56.51	67.89	48.59	57.53	77.65	9.81	46.60	14.28	63.90	89.59
4	25.23	49.26	8.97	53.73	20.43	60.06	24.76	47.22	53.94	13.89
5	21.76	12.70	36.57	47.24	30.40	50.10	21.28	3.46	51.81	29.47
6	14.66	46.95	32.86	36.51	9.30	19.87	35.59	51.28	37.41	48.30
7	16.10	54.24	79.07	28.97	87.46	43.01	31.70	75.45	48.00	74.91
8	27.64	84.65	7.27	45.93	50.70	24.70	54.80	20.44	36.32	55.35
9	8.06	29.04	53.12	43.26	77.77	36.06	28.40	58.16	29.82	21.40
10	18.62	56.35	6.63	38.58	44.65	50.71	22.60	78.11	20.29	39.25

* Where 3777 contains agar and BSS® Part I.

Table 2-7. Analysis of variance for trial 2 of artificial media comparison study to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	5444.97	9	605.00	21.81	< 0.0001	1.89
Treatment	7775.74	9	863.97	31.15	< 0.0001	1.89
Interaction	33350.12	81	411.73	14.84	< 0.0001	1.29
Error	19418.04	700	27.74			
Total	65988.88	799				

Table 2-8. Standard two-sample *t*-test for trial 2 of artificial media comparison study, assuming unequal variances comparing bite-second data for human subjects

	3777	8001	3777	8002	3777	8003	3777	8004	3777	8005
Mean	23.33	43.39	23.33	34.94	23.33	48.46	23.33	54.09	23.33	33.15
Variance	171.0	591.9	171.0	535.0	171.0	157.0	171.0	816.0	171.0	295.9
P(T<=t) one-tail	**0.02**		**0.09**		**0.0002**		**0.004**		**0.08**	

	3777	8006	3777	8007	3777	8008	3777	8009
Mean	23.33	32.77	23.33	39.72	23.33	47.60	23.33	43.28
Variance	171.0	290.4	171.0	731.0	171.0	295.4	171.0	579.8
P(T<=t) one-tail	**0.09**		**0.05**		**0.001**		**0.02**	

*Significant comparisons are bold.

**P-values are significant at α= 0.10, 0.05, 0.005, and 0.0005.

***Where 3777 contains agar and BSS® Part I.

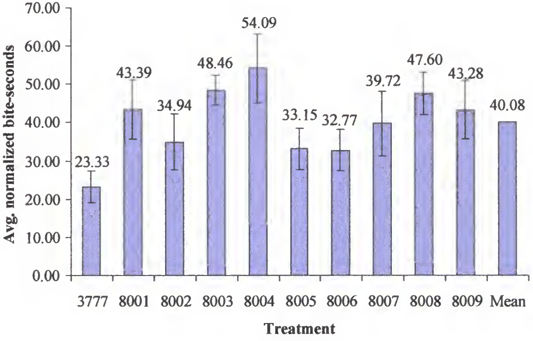


Figure 2-12. Normalized data graph for trial 2 of artificial media comparison study, showing the average bite-seconds (for ten repetitions) versus the overall mean, plus the standard error. Where 3777 contains agar and BSS® Part I.

Medium compound 3777 contained no attractants in any form. It was expected that human essence of any kind would significantly attract more mosquitoes than the untreated membrane. All nine subjects, even 8005 who is generally less attractive or the same as the untreated membrane, were significantly more attractive than no treatment. Since there were no subjects that were less attractive than the untreated membrane, it is possible that substances present on the skin identified by Bernier and Posey (2001) as mosquito inhibitors are also inhibitors of the attractants that are found on the skin (Bernier et al. 2000).

Trial 3

Ten repetitions were recorded for Trial 3. Data were normalized using the equation: SQRT of n + 1 (Table 2-9). Attractiveness was measured in average bite-seconds per repetition. Figure 2-13 shows the normalized data with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 2-10).

In this trial, seven of nine subjects were significantly more attractive than 3778 (agar and BSS$^{®}$ Plus)($\alpha = 0.01, p = 0.005$, $\alpha = 0.05, p = 0.014$, $p = 0.04$, $\alpha = 0.005, p = 0.004$, $p = 0.003$, $p = 0.003$, and $\alpha = 0.0001, p = 0.00007$), (Table 2-11).

Table 2-9. Normalized bite-second data for human subjects in trial 3 of artificial media comparison study

Repetition	3778	8001	8002	8003	8004	8005	8006	8007	8008	8009
1	12.9	47.0	30.4	19.2	21.9	12.4	16.6	71.3	42.3	10.6
2	6.1	18.0	23.6	12.2	21.3	25.5	25.4	75.7	49.7	24.8
3	14.7	71.3	27.4	46.2	54.9	27.8	15.8	93.5	46.1	48.0
4	29.6	18.0	37.7	73.2	29.8	34.1	21.5	37.4	43.2	71.0
5	33.7	59.0	13.1	35.5	13.5	57.4	2.8	89.4	29.6	38.3
6	44.8	47.4	55.2	29.2	50.7	29.5	10.4	102.8	23.2	23.4
7	19.7	45.7	43.4	65.0	22.9	17.0	20.2	74.3	39.2	13.5
8	10.2	37.0	32.2	52.7	32.9	18.9	54.6	63.1	53.7	16.3
9	6.3	42.9	49.4	36.4	43.0	9.6	40.0	64.3	72.1	33.3
10	10.9	12.6	48.8	51.2	42.2	12.0	24.9	14.0	7.2	43.7

* Where 3778 contains agar and BSS$^{®}$ Plus

Table 2-10. Analysis of variance for trial 3 of artificial media comparison study to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	3230.9	9	358.99	20.40	<0.0001	1.893
Treatment	18081.0	9	2009.00	114.10	<0.0001	1.893
Interaction	23298.0	81	287.63	16.34	<0.0001	1.293
Error	12320.0	700	17.60			
Total	56930.0	799				

Table 2-11. Standard two-sample *t*-test for trial 3 of artificial media comparison study, assuming unequal variances comparing bite-second data for human subjects

	3778	8001	3778	8002	3778	8003	3778	8004	3778	8005
Mean	18.9	39.9	18.9	36.1	18.9	42.1	18.9	33.3	18.9	24.4
Variance	168.5	357.1	168.5	174.1	168.5	373.7	168.5	191.7	168.5	202.4
P(T<=t) one-tail	**0.005**		**0.004**		**0.003**		**0.014**		0.19	

	3778	8006	3778	8007	3778	8008	3778	8009
Mean	18.9	23.2	18.9	68.6	18.9	40.6	18.9	32.3
Variance	168.5	218.6	168.5	701.9	168.5	313.8	168.5	347.3
P(T<=t) one-tail	0.25		**0.00007**		**0.003**		**0.04**	

*Significant comparisons are bold.

**P-values are significant at α= 0.01, 0.05, 0.005, and 0.0001.

***Where 3778 contains agar and BSS ® Plus.

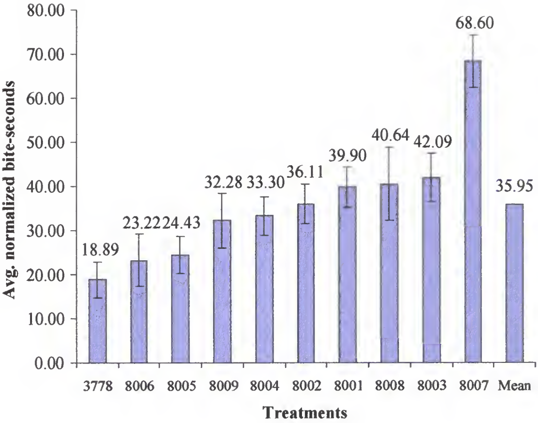


Figure 2-13. Normalized data graph for trial 3 of artificial media comparison study, showing the average bite-seconds (for ten repetitions) versus the overall mean, plus the standard error. Where 3778 contains agar and BSS® Plus.

These results show that seven out of nine subjects were more attractive than the untreated medium compound. Human essence present on the membranes (in most cases) was important in host attraction. This artificial host medium did have attractants present in the form of BSS® Part II, but that alone was not enough to attract mosquitoes away from human smell (Strauss et al. 1968).

Trial 4

Ten repetitions were recorded for trial 4. Data were normalized using the equation: SQRT of n + 1 (Table 2-12). Attractiveness was measured in average bite-seconds per repetition. Figure 2-14 shows the normalized data graph with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10$, $p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10$, $p < 0.0001$), (Table 2-13).

In this trial, three subjects were significantly less attractive than 3779 (blood, agar and BSS ® Part I) ($\alpha = 0.05$, $p = 0.03$, $\alpha = 0.10$, $p = 0.08$, $p = 0.09$), (Table 2-14).

Table 2-12. Normalized bite-second data for human subjects in trial 4 of artificial media comparison study

Repetition	3779	8001	8002	8003	8004	8005	8006	8007	8008	8009
1	14.7	47.1	42.3	30.3	60.1	50.8	24.4	69.3	70.2	6.7
2	86.3	33.1	32.5	60.6	11.1	23.5	20.7	44.2	15.6	15.5
3	44.6	40.2	53.8	32.0	20.5	28.1	27.5	101.3	37.9	58.6
4	60.2	115.7	36.4	9.5	85.5	1.3	35.2	28.8	31.3	27.6
5	27.3	74.9	18.0	72.3	101.3	38.1	6.6	92.5	43.0	56.1
6	58.6	27.8	54.3	21.8	54.5	20.4	38.7	89.0	68.2	34.8
7	58.6	36.5	53.1	25.1	25.2	30.6	112.1	26.9	20.7	33.2
8	11.6	54.1	28.5	46.1	63.2	15.3	45.3	23.3	22.1	44.8
9	81.8	16.3	56.8	24.2	44.6	35.7	34.2	67.3	59.3	53.3
10	49.0	80.2	56.2	22.2	68.8	60.4	56.5	58.0	58.3	21.4

* Where 3779 contains blood, agar and BSS ® Part I.

Table 2-13. Analysis of variance for trial 4 of artificial media comparison study to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	2227.87	9	247.54	2.82	0.0029	1.89
Treatment	9184.23	9	1020.47	11.61	<0.0001	1.89
Interaction	45085.58	81	556.61	6.33	<0.0001	1.29
Error	61514.52	700	87.88			
Total	118012.21	799				

Table 2-14. Standard two-sample *t*-test for trial 4 of artificial media comparison study, assuming unequal variances comparing bite-second data for human subjects

	3779	8001	3779	8002	3779	8003	3779	8004	3779	8005
Mean	49.26	52.60	49.26	43.18	49.26	34.39	49.26	53.49	49.26	30.42
Variance	649.0	888.9	649.0	188.9	649.0	377.3	649.0	828.4	649.0	292.8
P(T<=t) one-tail	0.40		0.26		**0.08**		0.37		**0.036**	

	3779	8006	3779	8007	3779	8008	3779	8009
Mean	49.26	40.14	49.26	60.06	49.26	42.65	49.26	35.20
Variance	649.0	827.5	649.0	822.9	649.0	413.3	649.0	317.0
P(T<=t) one-tail	0.23		0.19		0.26		**0.086**	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.05$ and 0.10.

***Where 3779 contains blood, agar and BSS ® Part I.

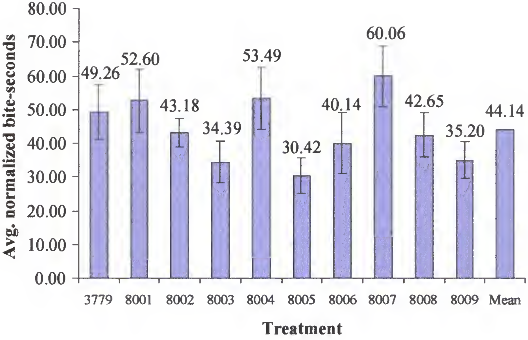


Figure 2-14. Normalized data graph for trial 4 of artificial media comparison study, showing the average bite-seconds (for ten repetitions) versus the overall mean, plus the standard error. Where 3779 contains blood, agar and BSS® Part I.

In this trial as well as the first, both artificial media compounds contained blood with 3779 missing the attractants (BSS® Part II) found in 3776. Compounds found within the red-blood cells are phagostimulatory, however these compounds can not be sensed by insects while locked inside the cell. Insects must use other methods to determine which host to "taste" (Galun et al. 1985). Results suggest that without the addition of non-blood attractants mosquitoes did not preferentially feed upon any one subject, however, there were more subjects that were less attractive than the untreated host. This could be due to the addition of previously mentioned inhibitors found in larger amounts on some subjects. It is possible that lower amounts of inhibitors present on the skin of some subjects are more noticeable without non-blood attractants present in the medium (Strauss et al. 1968, Bernier and Posey 2001).

Comparison of Artificial Host Media

Untreated media compound data were pulled from each trial and compared to each other (Table 2-15). This comparison is justified because all other aspects of each trial were constant with only the media compound changing. Attractiveness was measured in average bite-seconds per repetition. Figure 2-15 shows the normalized data graph with the average bite-seconds for each treatment and the overall mean.

There was a significant difference between 3776 (blood, agar and BSS® Plus) and 3777 (agar and BSS® Part I) as well as 3778 (agar and BSS® Plus), but no significant difference between 3776 and 3779 (blood, agar and BSS® Part I) ($\alpha = 0.05, p = 0.03, p = 0.01, p = 0.40$). There was also a significant difference between 3777 and 3779 ($\alpha = 0.01$, $p = 0.007$) and 3778 and 3779 ($\alpha = 0.005, p = 0.003$). There was no significant difference between 3777 and 3778 ($p = 0.23$) (Table 2-16).

Table 2-15. Comparison of bite-second data for untreated media compounds combined from previous trials

Repetition	3776	3777	3778	3779
1	16.35	26.19	12.88	14.66
2	89.23	18.57	6.10	86.29
3	88.49	56.51	14.75	44.62
4	16.90	25.23	29.61	60.21
5	13.40	21.76	33.68	27.32
6	15.54	14.66	44.76	58.62
7	41.73	16.10	19.68	58.58
8	84.89	27.64	10.22	11.60
9	61.40	8.06	6.33	81.77
10	34.19	18.62	10.95	48.95
Mean	46.21	23.33	18.89	49.26

*Where 3776 contains blood, agar and BSS® Plus, 3777 contains agar and BSS® Part I, 3778 contains agar and BSS® Plus, and 3779 contains blood, agar and BSS® Part I.

Table 2-16. Standard two-sample *t*-test for artificial media, assuming unequal variances comparing bite-second data for media compounds

	3776	3777	3776	3778	3776	3779
Mean	46.21	23.33	46.21	18.89	46.21	49.26
Variance	1033.05	170.99	1033.05	168.49	1033.05	649.03
P(T<=t) one-tail	**0.03**		**0.01**		0.41	

	3777	3778	3777	3779	3778	3779
Mean	23.33	18.89	23.33	49.26	18.89	49.26
Variance	170.99	168.49	170.99	649.03	168.49	649.03
P(T<=t) one-tail	0.23		**0.007**		**0.003**	

*Significant comparisons are bold.

**P-values are significant at α= 0.05, 0.01, and 0.005.

***Where 3776 contains blood, agar and BSS® Plus, 3777 contains agar and BSS® Part I, 3778 contains agar and BSS® Plus and 3779 contains blood, agar and BSS® Part I.

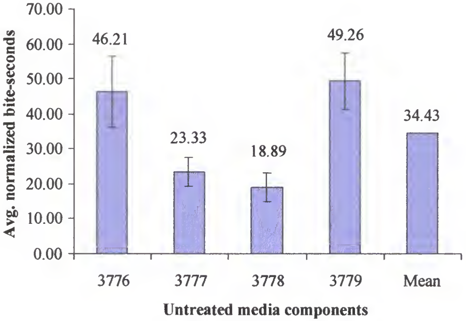


Figure 2-15. Normalized data graph for untreated media components, showing the average bite-seconds (for ten repetitions) versus the overall mean, plus the standard error. Where 3776 contains blood, agar and BSS® Plus, 3777 contains agar and BSS® Part I, 3778 contains agar and BSS® Plus and 3779 contains blood, agar and BSS® Part I.

These results show that the addition of blood to an artificial medium is important in the stimulation of feeding. In our study there was no significant difference between the media compounds that contained blood and no significant difference between the two compounds that did not contain blood. Without the addition of human essence these compounds can only stimulate feeding based upon phagostimulants found within the media, and while buffered solution may improve feeding in some species, it is not effective alone (Galun et al. 1984). Although studies have shown that insects are not chemoreceptive to blood alone, this study shows no difference in media that contains only blood versus media with blood and non-blood attractants (Galun et al. 1985). There is a possibility that some masking effects from hosts or non-blood attractants do occur using an artificial medium with both blood and non-blood attractants added (Braverman et al. 1991). When only blood was added to the media more subjects show repellent tendancies than when blood and non-blood attractants were added. Possibly the non-blood attractants overpower naturally occurring inhibitors found on human skin (Bernier and Posey 2001), in the presence of CO_2 (Bar-zeev et al. 1977, Schreck et al. 1990, Cosgrove and Wood 1995).

CHAPTER 3
PREFERENTIAL HOST SELECTION

Introduction

Through out history, people have passed along tales of individuals that are repellent or rarely bitten by mosquitoes (Maibach et al. 1966C). And in fact, Pickett and Woodcock (1996) suggest that, in cattle herds that only contain one species of cattle, some individuals are not as susceptible to hematophagous Diptera as others, and that these individuals probably produce something that covers the usual attractants produced by each specific breed of cattle. Conversely, there would be a few individuals within the herd that would be more susceptible to the attentions of unwanted hematophagous insects. These few individuals would, in theory, provide a measure of protection for the rest of the herd by effectively drawing the majority of the blood feeders from the rest of the herd to feed on the "attractive" individuals. The kairomones or allomones involved may be used by the insects to identify hosts, which possibly express immunological responses that could be unsuitable for the insect's particular needs. This theory of individual cattle being more repellent than others could be transcribed to human attraction and repellency. If the particular chemicals involved could be isolated and synthesized, there is a possibility that a more effective method of controlling mosquito-vectored diseases could be created (Okine 1994). Recently Bernier et al. (1999) described over 275 compounds found on human skin that act as attractants and at least one that acts as an inhibitor of mosquito attraction (Bernier and Posey 2001).

Field data have consistently proven that insects do attack certain individuals more than others. One study found consistent statistical differences in the attraction levels of five different human subjects to *Simulium venustum* (s.l.) over a two-year period. The attraction was measured over a distance and was based primarily on exhaled breath odors, with variables (such as type of soap) being held the same for some of the tests. These results confirmed the common belief that some people are more likely to be bitten by black flies (Schofield and Sutcliffe 1996). More recently, studies of human biting catches in Africa have shown that certain individuals attracted more *Anopheles gambiae* s.l. than others consistently for more than two months (Brady et al. 1997). Lindsay et al. (1993), found that attractant subjects remained so for more than 2 years, showing that the phenomenon was not temporary, but remained relatively stable.

This study will attempt to determine whether mosquitoes, specifically colony reared *Aedes aegypti*, show individual human host preference in selection of a blood meal within the confines of a 10-choice olfactometer and will attempt to assign designations of attractant or repellent properties to individual subjects.

Methods and Materials

Mosquitoes Used in Trial

Mosquitoes used were handled with the same techniques as described in Chapter Two.

Colony

Mosquitoes were kept in colony as described in Chapter Two.

Olfactometer

The olfactometer used is described in the materials and methods of Chapter Two.

Artificial Host

The standard (3776) artificial host medium was used in this trial with the same methods of treating the membrane as described in Chapter Two.

Volunteer Information

The same volunteers and methods were used for both Chapters Two and Three trials.

Membrane Preparation

The membranes were prepared as outlined in Chapter 2, using the base medium, 3776 (blood, agar, and BSS® Plus) in the artificial host container.

Data Recording

As outlined in Chapter Two.

Procedure

The trial consisted of thirteen eight-hour replications (individual tests run once or twice a day, during at least one prime feeding time, comparing subjects). Replications were used in the final statistical analysis if they had no mechanical, electrical or behavioral complications, such as amplifiers not communicating with the computer or sensors shorting, insects trapped behind sensors or no activity recorded for the standard (3776), during a replication.

Volunteers were recruited as outlined in Chapter Two, with nine being chosen for this trial.

Statistical Analysis

Data were analyzed as outlined in Chapter Two.

Results

Thirteen repetitions were recorded for this trial. Of the thirteen repetitions, three were eliminated using the following criteria. Replications whose total number of bite-seconds was more or less than 50% different than the trial average were eliminated. Replications with a low average activity implied poor mosquito quality and replications with a high average activity implied insects were trapped underneath sensors, in effect completing the circuit continuously (Table 3-1).

Table 3-1. Average number of bite-seconds for eight-hour exposures (raw data) for *Ae. aegypti* host preference trials

Replication	Subject Number										Total / rep
	3776	8001	8002	8003	8004	8005	8006	8007	8008	8009	
1	266	3831	6008	863	708	755	330	4527	5467	524	23280
2	7961	170	863	3150	5651	3041	1065	8884	8030	8435	47249
3	7830	470	715	260	341	1242	3381	3126	104	424	17891
4*	386	12319	10108	2281	902	2375	1918	6286	8753	8689	**54016**
5	285	2142	1169	611	81	614	997	5955	4306	174	16334
6*	2818	1941	1868	2197	2363	3999	4640	12741	11384	4328	**48278**
7	179	5299	3402	2329	3546	116	112	4191	433	305	19911
8	240	448	690	3792	1260	706	11087	470	1771	135	20600
9*	12364	3450	5528	11896	4731	1237	2372	6006	14416	3919	**65919**
10	1740	2302	2019	1251	120	225	691	9649	203	3351	21551
11	7205	1051	1410	21	4768	254	3183	5505	893	2064	26356
12	3769	2966	2362	320	2451	196	8501	9317	2712	1047	33640
13	1168	4602	1348	2659	3877	854	220	3246	4338	639	22951
Average	3555	3153	2884	2433	2369	1201	2961	6146	4832	2618	32152
									Average + 50%		***48228***
									Average - 50%		***16076***

*Replications were eliminated due to extreme high or low averages. For example, in this trial, replications 4, 6 and 9 were eliminated because their averages were greater than the overall average plus 50%.

** Where 3776 is an artificial media containing blood, agar and BSS® Plus.

Attractiveness was measured in average bite-seconds per repetition. Figure 3-1 shows the normalized data with the average bite-seconds for each subject and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 3-2).

Table 3-2. Analysis of variance for human subjects trial to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	8625.589	9	958.3988	20.78272	< 0.0001	1.893241
Treatment	12274.49	9	1363.832	29.57447	< 0.0001	1.893241
Interaction	44083.48	81	544.2405	11.80177	< 0.0001	1.293221
Error	32280.63	700	46.11518			
Total	97264.18	799				

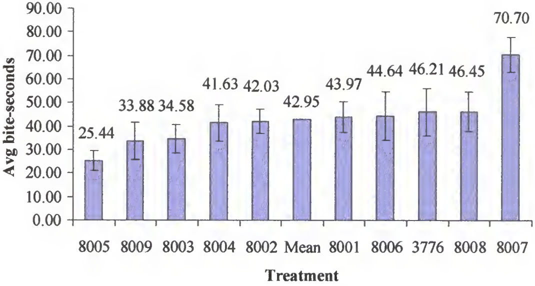


Figure 3-1. Normalized data graph for host preference trial, showing the average bite-seconds (for ten repetitions) plus the standard error. Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Treatments could be grouped into one of three groups. Group 1 was not significantly different than the standard (3776), group 2 was significantly more attractive than 3776, and group 3 was significantly less attractive than 3776 (Table 3-3). Subject 8005 was significantly less attractive than 3776 ($\alpha = 0.05, p = 0.04$) and subject 8007 was significantly more attractive than 3776 ($\alpha = 0.05, p = 0.03$). All other subjects were statistically the same as 3776 (Table 3-4).

Table 3-3. Human subject treatment groups

Group 1	Group 2	Group 3
8001	8007	8005
8002		
8003		
8004		
8006		
8008		
8009		

Table 3-4. Standard two-sample *t*-test assuming unequal variances comparing subjects

	3776	8001	3776	8002	3776	8003	3776	8004	3776	8005
Mean	46.21	43.97	46.21	42.03	46.21	34.58	46.21	41.63	46.21	25.44
Variance	1033.05	439.71	1033.05	258.60	1033.05	367.21	1033.05	609.29	1033.05	171.36
P(T<=t) one-tail	0.43		0.36		0.17		0.36		0.04	

	3776	8006	3776	8007	3776	8008	3776	8009
Mean	46.21	44.64	46.21	70.70	46.21	46.45	46.21	33.88
Variance	1033.05	1072.71	1033.05	543.21	1033.05	743.51	1033.05	625.77
P(T<=t) one-tail	0.46		0.03		0.49		0.18	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.05$.

***Where 3776 is an artificial media containing blood, agar and BSS® Plus.

Discussion

Most olfactometer testing measures the orientation attraction of mosquitoes to a host or chemical. The mosquitoes are counted as they enter or stay in a treatment chamber (Kline 1998, Bernier and Posey 2001). The UF/IFF olfactometer actually measures feeding or probing activity (Butler and Katz 1987). This type of system allows a more-in-depth look at group dynamics and actual feeding preference versus basic orientation towards a host in the presence of IR, moisture, and intermittent CO_2. This system also allows for the testing of multiple subjects at one time, therefore the mosquitoes have more options for feeding (McKenzie 2000, Butler and Katz 1987).

As previously reported (McKenzie 2000) in a group of approximately ten people there will be at least one person who draws the mosquitoes and one who repels them. In this trial, this hypothesis was supported by the results. One subject was significantly more attractive than the remainder of the group and one subject was significantly less attractive than the others. Why this occurs has been the subject of much research (Acree et al. 1968, Bar-zeev et al. 1977, Bernier et al. 1999, 2000, Braks and Takken 1999). In the past it was believed that people only showed different levels of attraction, but more recently (Bernier and Posey 2001) it has been shown that not only do people secrete chemicals that cause attraction, but also inhibition. Many methods have been used to collect and test human secretions for attractancy, the most common of which is the use of glass beads (Carlson et al. 1992, Bernier et al. 1999, 2000, 2002). Our research has shown that a silicone matrix is also effective in maintaining human secretions for testing (McKenzie 2000).

The subjects in this trial did not have agreement between self-ratings and actual olfactometer ratings. For example, the significantly "attractive" subject was self rated as "rarely attractive" and the significantly "repellent" subject was self rated (based on previous olfactometer trials) as "very attractive". This divergence could be due to several possibilities, such as, being bitten by different species of mosquito while in the field, masking by host presence or possibly current medications (Braverman et al. 1991).

The species of mosquito that was used in this trial is anthropophilic and a transmitter of disease; it is also easy to maintain in colony. These are the primary reasons why *Ae. aegypti* is used in many attraction studies. However, it is an extremely rare species to find in Florida, and it has been replaced throughout most of Florida by *Aedes albopictus* (Reeves and Milby 1989, O'Meara et al. 1995).

The principle of group dynamics as applied here determines a person's position within a group being relative to the dynamics of the group. Braverman et al. (1991) determined that the addition of new subjects may shift attraction ratings within a group. For example, in this sample group, subject 8007 was extremely attractive to mosquitoes, however, within 8007's familial group or normal peers, some other person may be more attractive, thus drawing mosquitoes away from 8007.

Chemical interactions within a host may cause a change in level of attractiveness. For instance, Shirai et al. (2002) have shown that the ingestion of alcohol increases a host's attraction to mosquitoes. The shift in attraction is probably due to unknown chemicals found on the skin or breath after ingestion. Similarly, medications given to control high blood pressure may cause an increase in attraction levels, whereas medications to control high cholesterol may cause a decrease in attraction levels (McKenzie 2000 and Butler personal communication). Subject 8005 was used in previous studies testing human subjects and attraction levels. This particular subject was consistently rated very attractive even when placed in different groups. From the previous testing (1999 and 2000) to the current testing (2002 and 2003) one of the only differences in 8005 was the addition of systemic anti-cholesterol medication. This, of course, does not prove that anti-cholesterol medication is a "systemic" repellent, but in two different studies with two different subjects, the addition of a particular brand of anti-cholesterol medication dropped attraction levels significantly, indicating the resulting attractancy profile had shifted. Clinical studies need to be evaluated with good controls and accurate testing to determine attractancy levels before and after drug treatment.

CHAPTER 4
ANALYSIS OF COLLECTED HUMAN ESSENCE BY GAS CHROMATOGRAPHY

Introduction

After determining a significant difference between two subjects and the standard in Chapter Three, further investigation was initiated. Subject 8005 was significantly less attractive than the standard (3776- blood, agar and BSS® Plus) and subject 8007 was significantly more attractive than 3776. In this study the term “repellent” has been defined as significantly less attractive than 3776 and the term “attractant” has been defined as significantly more attractive than 3776. With these definitions in mind, the subjects will be referred to as attractant or repellent.

In standard chemical attractant trials based on the standard 3776, I hypothesized that attractants work better than 3776 because of the presence of something on the membrane. I predict that the presence of human essence of any kind, versus nothing, would attract more mosquitoes every time, but such is not the case. Because mosquitoes are not actually attracted to blood, this implies that not only is there something present on people who are attractive to mosquitoes, but also the addition of something on people who are repellent to mosquitoes (Klowden 1995, Bernier and Posey 2001).

Anthropological studies as well as criminal studies have shown that individual humans have distinctive odors (unless they are identical twins). There may be small variations in that odor depending upon diet and perfumes, yet basically it remains the same. This is evidenced by the fact that bloodhounds and other dogs are used to locate an individual from an unwashed article of clothing or some other personal item that was not necessarily worn on the day of the search. Skin secretions, the basis of "body odor," are fairly consistent and, as in some species of New World monkeys, body secretions are probably unique to each individual (unlike breath odors) which are constantly changing (Sommerville et al. 1986).

When testing body secretions in a gas chromatograph (GC), water becomes a problem. Because of the presence of water in human secretions many methods have been developed to extract the "essence" of the human. Primarily, extracts of human handled glass have been used. This method has led to chromatogram profiles of extracted human essence that are usually similar. Normally the subjects will differ only in the quantity of certain components of the profile, which has been an important point relative to mosquito host location and host preference. Recently, a lot of attention has been placed on identifying the compounds found in human essence after various methods of extraction. Over 300 compounds have been identified, and the methods used have influenced the number and amount of compounds isolated (Bernier et al. 1999). Of those 300 compounds identified, 277 were shown to be attractants for *Aedes aegypti*. Even with this large number of attractant compounds isolated from human extracts, there is no way to state that "all" attractant compounds have been identified. More testing needs to be done, comparing subjects who are at different ranges of the attractant spectrum (Bernier et al. 1999, 2000).

The purpose of this study was to use gas chromatography to determine if there was a different profile of peaks between samples from subjects that had been previously separated as attractant and repellent by olfaction trials, not to determine the identity of the components of each sample.

Methods and Materials

Sample preparation for GC analysis. Subjects 8005 and 8007 were instructed to each wear a membrane for four hours, according to the methods for the olfactometer assay from the previous chapter. As per methods in Chapter Two and Three, at the end of the four hours each membrane was removed from the subject, cut into four pieces and placed in a vial for storage. One piece of untreated membrane (no human contact) and one piece from each subject were placed in small vials with just enough hexane (approximately 1.0 mL) to cover each tightly rolled membrane. The membranes were allowed to soak for 24 hours, and then were removed from the vial. The remaining hexane was concentrated with a nitrogen stream. Each sample was analyzed twice. Sample membrane from subject 8007 was analyzed twice on day one due to a low dose on the first analysis. A 1.0 –μL sample was injected into an AT1 instrument with a non-polar column (25 m x 0.25 μm, catalog # 932525). The column was heated from 200 to 300° C at a rate of 4° C/ minute. This instrument used helium carrier with a gas flow rate of 25 meters in 1.91 minutes or 21.8 cm/ second. A standard hydrocarbon sample was prepared from commercially available synthetic hydrocarbons (Sigma chemical company). The standard contained even straight chain hydrocarbons from C20 to C34 and was run prior to running the first trial (Appendix B and C). During the second trial, cholesterol and campesterol standards were run prior to running the samples (Appendix D). The data and resulting chromatograms were recorded and processed with a Hewlett Packard 3390A integrator. Retention Indices (RI) were calculated (for all peaks with retention times falling between Carbon standards) based on Kovat's equation and the C20 to C34 standards.

Results

The first trial compared the untreated membrane, 8005, 8007, and a re-run of 8007 (GC tables can be found in Appendix C). Overall there were 67 peaks with retention times between 2.36 and 39.16 min (Table 4-1). The retention times of all samples were averaged and plotted against the RI values, to show missing or additional peaks (Figure 4-1). The untreated sample had 3 peaks (between 30 and 34 carbons in length) that were not found in any other sample, was missing 1 peak (28 carbons) that was found in all other samples, and was missing 6 peaks (between 26 and 31 carbons in length) that were also missing in one other sample. Sample 8005 had 10 peaks (3 were < 20 carbons in length, 6 were between 26 and 33 carbons in length, and 1 was > 34 carbons in length) that were not found in any other sample and was missing 1 peak (> than 34 carbons in length) that was missing from one other sample. Sample 8007 had 7 peaks (3 were between 29 and 33 carbons in length and 4 were > than 34 carbons in length) that were not found in any other sample, was missing 11 peaks (9 between 20 and 33 carbons in length and 2 > than 34 carbons in length) that were found in all other samples, and was missing 8 peaks (7 between 26 and 31 carbons in length and 1 > 34 carbons in length) that were missing from one other sample. The re-run sample of 8007 had 4 peaks (3 between 21 and 25 carbons in length and 1 > 34 carbons in length) that were not found in any other samples and was missing 1 peak (27 carbons) that was missing from one other sample (Table 4-2).

Table 4-1. Combined average retention times (RT) and sample retention indices (RI) for first trial

Peak #	Sample Avg RT (min)	Untrtd (RI)	8005 (RI)	8007 (RI)	8007R (RI)
1	2.36	n/c	n/c	n/c	n/c
2	2.54	-	n/c	-	-
3	2.80	n/c	n/c	n/c	n/c
4	3.13	-	n/c	-	-
5	3.54	n/c	n/c	n/c	n/c
6	4.67	n/c	n/c	n/c	n/c
7	6.15	-	n/c	-	-
8	6.25	2006	2005	2005	2006
9	7.36	2089	2089	-	2089
10	7.78	-	-	-	2121
11	8.28	2160	2159	2158	2160
12	8.75	2195	2195	2193	2195
13	10.27	2291	2292	2291	2292
14	10.61	2313	2313	2312	2313
15	10.79	-	-	-	2324
16	11.90	2394	2394	2394	2395
17	13.11	2465	2466	2464	2466
18	13.59	2493	2493	2493	2493
19	13.71	-	-	-	2500
20	14.68	2556	2557	-	2557
21	15.31	2593	2594	2593	2594
22	15.69	2616	2616	2615	2616
23	16.42	-	2658	-	2659
24	16.58	-	2668	-	-
25	17.03	2694	2695	2694	2695
26	18.13	2758	2759	-	-
27	18.25	2766	2767	2764	2767
28	18.45	-	2778	-	-
29	18.74	2795	2795	2794	2795
30	18.90	-	2805	2803	2805
31	19.62	-	2847	-	-
32	19.75	2854	2855	-	2855
33	20.00	2869	2870	-	2870
34	20.17	2880	2880	2879	2881
35	20.35	-	2891	-	-
36	20.43	2895	2896	2895	2896
37	20.67	-	-	2910	-
38	20.72	2913	2914	-	2913
39	20.93	-	2926	-	2926
40	21.21	-	2943	-	2943
41	21.42	-	2955	-	2955
42	21.67	2969	2970	-	2970
43	21.87	2981	2982	-	2982
44	22.06	2993	2994	2992	2995
45	22.53	3022	3023	-	3022
46	23.05	3055	3057	3052	3055
47	23.54	-	3085	-	3084
48	23.70	3095	3096	3093	3096
49	24.18	-	3125	-	3125
50	24.31	3133	-	-	-
51	24.76	3161	-	-	-
52	25.28	3193	3194	3190	3194
53	25.90	-	3227	-	-
54	27.25	3299	-	-	-
55	27.44	-	-	3309	-
56	27.65	-	-	3321	-
57	27.73	3324	3326	-	3324
58	28.97	-	3391	-	-
59	30.59	-	-	n/c	-
60	30.69	n/c	n/c	-	n/c
61	34.28	-	-	n/c	-
62	34.40	n/c	-	-	n/c
63	34.45	-	n/c	-	-
64	38.12	-	-	n/c	-
65	38.25	-	-	-	n/c
66	39.00	-	-	n/c	-
67	39.16	n/c	n/c	-	n/c

*n/c could not be calculated with Carbon standards used

** - denotes peak not present

A

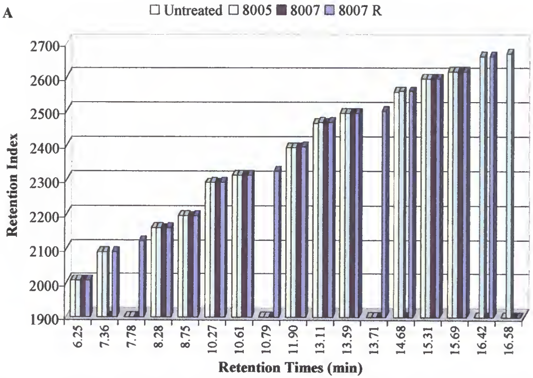


Figure 4-1a-c. Trial 1. Combination of untreated membrane and samples 8005, 8007 and the re-run 8007. Average retention times versus the retention index of samples. Peaks with less than 20 carbons or more than 34 carbons were not plotted. A) Retention times of 6.25 to 16.58 min. B) Retention times of 17.03 to 21.42 min. C) Retention times of 21.67 to 28.97 min.

B

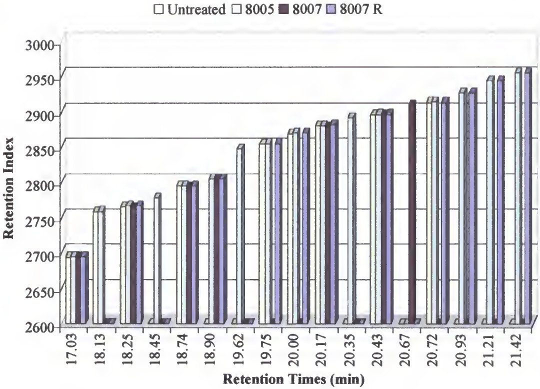


Figure 4-1. Continued

C

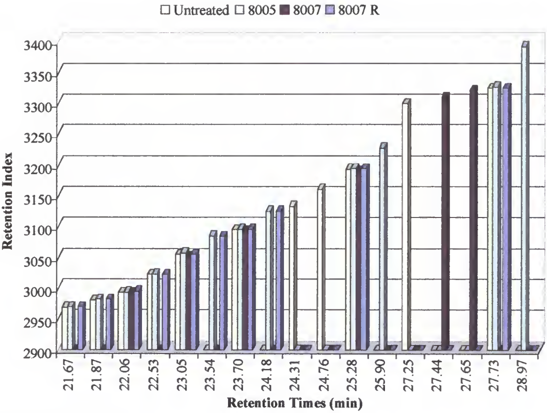


Figure 4-1. Continued

Table 4-2. Peaks that are either unique or missing from one or more samples

Peak #	Sample Avg RT (min)	Untreated (RI)	8005 (RI)	8007 (RI)	8007 R (RI)
2	2.54	-	n/c	-	-
4	3.13	-	n/c	-	-
7	6.15	-	n/c	-	-
9	7.36	2089	2089	-	2089
10	7.78	-	-	-	2121
15	10.79	-	-	-	2324
19	13.71	-	-	-	2500
20	14.68	2556	2557	-	2557
23	16.42	-	2658	-	2659
24	16.58	-	2668	-	-
26	18.13	2758	2759	-	-
28	18.45	-	2778	-	-
30	18.90	-	2805	2803	2805
31	19.62	-	2847	-	-
32	19.75	2854	2855	-	2855
33	20.00	2869	2870	-	2870
35	20.35	-	2891	-	-
37	20.67	-	-	2910	-
38	20.72	2913	2914	-	2913
39	20.93	-	2926	-	2926
40	21.21	-	2943	-	2943
41	21.42	-	2955	-	2955
42	21.67	2969	2970	-	2970
43	21.87	2981	2982	-	2982
45	22.53	3022	3023	-	3022
47	23.54	-	3085	-	3084
49	24.18	-	3125	-	3125
50	24.31	3133	-	-	-
51	24.76	3161	-	-	-
53	25.90	-	3227	-	-
54	27.25	3299	-	-	-
55	27.44	-	-	3309	-
56	27.65	-	-	3321	-
57	27.73	3324	3326	-	3324
58	28.97	-	3391	-	-
59	30.59	-	-	n/c	-
60	30.69	n/c	n/c	-	n/c
61	34.28	-	-	n/c	-
62	34.40	n/c	-	-	n/c
63	34.45	-	n/c	-	-
64	38.12	-	-	n/c	-
65	38.25	-	-	-	n/c
66	39.00	-	-	n/c	-
67	39.16	n/c	n/c	-	n/c

*n/c could not be calculated with Carbon standards used

** - denotes peak not present

The second trial compared the untreated membrane, 8005 and 8007 (GC tables can be found in Appendix C). Overall there were 47 peaks with retention times between 2.27 and 44.33 min. The untreated sample was missing 2 peaks (1 at 31 carbons and 1 > 34 carbons in length) that were found on all other samples. Sample 8005 had 9 peaks (between 25 and 32 carbons in length) that were not found on any other sample. Sample 8007 had 4 peaks (3 between 24 and 32 carbons in length and 1 > 34 carbons in length) that were not found on any other sample and was missing 1 peak (20 carbons) that was found in all other samples (Table 4-3). There were 13 peaks found on sample 8005 and/or sample 8007 that were not found on the untreated standard (Table 4-4). The retention times of all samples were combined and plotted against the RI values, to show missing or additional peaks (Figure 4-2).

Table 4-3. Combined average retention times (RT) and sample retention indices (RI) for second trial

Peak #	Sample Avg RT (min)	Untrtd (RI)	8005 (RI)	8007 (RI)
1	2.27	n/c	n/c	n/c
2	2.70	n/c	n/c	n/c
3	3.42	n/c	n/c	n/c
4	4.52	n/c	n/c	n/c
5	6.08	n/c	n/c	n/c
6	7.17	2074	2075	-
7	8.08	2143	2144	2144
8	8.53	2177	2178	2178
9	10.04	2276	2277	2278
10	10.38	2298	2298	2298
11	11.65	2378	2379	2379
12	12.86	2450	2451	2451
13	13.33	2477	2478	2479
14	13.45	-	-	2485
15	14.42	-	2542	-
16	15.04	2577	2578	2578
17	15.43	2600	2601	2601
18	16.14	-	2642	-
19	16.76	2677	2679	2679
20	17.84	-	2742	-
21	17.98	2750	2750	2750
22	18.46	2778	2778	2779
23	18.63	-	-	2788
24	19.48	-	2839	-
25	19.70	2851	2852	2853
26	19.88	2862	2863	2863
27	20.14	2878	2878	2879
28	20.44	2896	2896	2896
29	20.91	-	2925	-
30	21.15	-	2939	-
31	21.37	-	2952	-
32	21.57	2963	2964	2965
33	21.77	2975	2976	2977
34	22.24	3004	3004	3006
35	22.77	3037	3038	3038
36	23.23	-	3066	-
37	23.41	3076	3077	3078
38	24.03	-	3115	3116
39	24.99	3175	3175	3176
40	25.63	-	3213	-
41	26.40	-	-	3254
42	27.35	3305	3305	3305
43	30.22	n/c	n/c	n/c
44	33.82	n/c	n/c	n/c
45	36.67	-	-	n/c
46	38.40	n/c	n/c	n/c
47	44.33	-	n/c	n/c

*n/c could not be calculated with Carbon standards used

** - denotes peak not present

Table 4-4. Additional peaks found on samples 8005 or 8007 that were not found on untreated membrane

Peak #	Sample Avg RT	Untrtd (Area)	8005 (Area)	8007 (Area)
14	13.45	-	-	2485
15	14.42	-	2542	-
18	16.14	-	2642	
20	17.84	-	2742	-
23	18.63	-	-	2788
24	19.48	-	2839	-
29	20.91	-	2925	-
30	21.15	-	2939	-
31	21.37	-	2952	-
36	23.23	-	3066	-
40	25.63	-	3213	-
41	26.40	-	-	3254
45	36.67	-	-	n/c

*n/c could not be calculated with Carbon standards used

** - denotes peak not present

The standard cholesterol sample was run twice and the standard campesterol sample was run three times (Appendix E). The retention times and RI values with standard deviation were averaged and recorded in Table 4-5.

In the first trial the untreated standard, sample 8005, and sample 8007R each contained a peak that had a RI value that was consistent with the average RI value of cholesterol ± 1 standard deviation (sd). There were no peaks in any sample with RI values consistent with campesterol's average RI value.

In the second trial there were no peaks consistent with the average RI values for either sterol standard.

Table 4-5. The average retention times and average area for sterol standards

Peak #	Standard	RT (min)	RI	Standard Deviation
1	Cholesterol	23.10	3057	± 3.07
2	Campesterol	24.84	3166	± 1.90

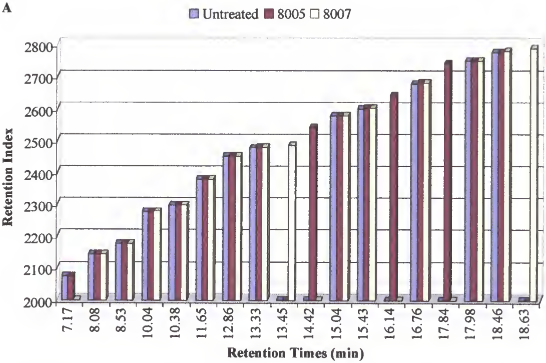


Figure 4-2a-b. Trial 2. Combination of untreated membrane and samples 8005, 8007. Average retention times versus the Retention Index of samples. Peaks with less than 20 carbons or more than 34 carbons were not plotted. A) Retention times of 7.17 to 18.63 min. B) Retention times of 19.48 to 27.35 min.

B

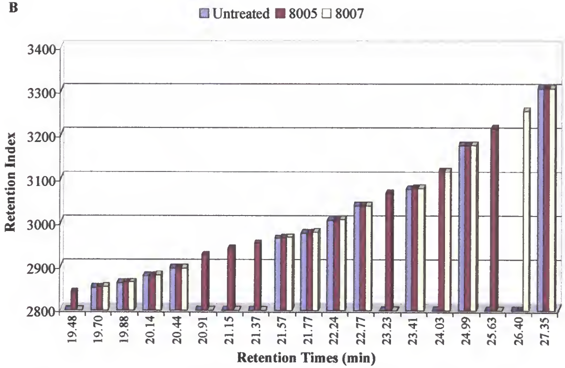


Figure 4-2. Continued.

Discussion

The body secretions extracted from mammals are a complex blend of compounds that can indicate health, age, gender and diet, any of which could signal that a host is a suitable source of nutrition for a mosquito. "The study of complete host odor blends is essential to fully understand the potentially complex role of individual compounds in attraction or repellence during vector host location" (McCall et al. 1996, p. 177).

There were differences in the untreated sample versus the human samples. Some peaks were not present on the human samples, but were present on the untreated sample. This may have occurred because of a combination of factors. Improper handling techniques, loss of compound in washing procedure, or possibly the presence of something on the treated samples that was able to bind with the compound from the untreated sample, making the compound "disappear" from human samples. Bernier and Posey (2001) have identified at least one chemical (patent pending) found on human skin that inhibits mosquito attraction. There may be more compounds that inhibit attractants or other substances found on skin. More research needs to be done to determine what compounds are represented on test subjects and if the compounds identified cause any distinct behavior in bioassays. Cholesterol has been identified as a component of human skin emanations and even though it is present in large amounts, does not appear to affect mosquito behavior (Bernier et al. 2002). In our first trial cholesterol was found in almost every sample assayed, but was not found in any of the samples run in the second trial. This absence is probably due to the age of the sample. In the first trial samples were analyzed on the same day as collection; in the second trial the samples were a couple of days old. Cholesterol could have been broken down quickly by bacteria present in the sample.

There were differences between human subjects in my tests. These human subjects had been identified previously as significantly different from each other and from the standard in bioassay tests. The differences in the chromatogram profiles and relative quantities for peaks with the same retention times prove that there was a difference between the two human samples tested, as well as the standard. This study was a preliminary study only, and further testing will be needed in order to determine if these differences are repeatable. However, because there were unique peaks found in all samples and especially in the two human samples, this study preliminarily supports the original hypothesis that to be an attractant or a repellent, something must be added to the base line (Klowden 1995).

Gas chromatography is very useful in identification of components of skin emanations (Bernier et al. 2000, Bernier et al. 2001, Bernier et al. 2002). Glass has been the collection material of choice in past assays of skin emanations (Bar-zeev et al. 1977, Bernier et al. 1999). However, in this study a silicone matrix was used to collect skin emanations for bioassay in the UF/IFF olfactometer. After successful bioassay results, the same matrix was used to collect emanations for GC analysis. Bernier et al. (2002), collected skin emanations from humans he had previously identified as "more attractive" and "less attractive" using glass beads held in the palm of the hand. The emanations were tested by gas chromatography- mass spectrometry. Differences in subjects were found, however, the differences consisted primarily of differing relative peak size and not numbers of peaks present. In my study, there were many differences in peak number as well as relative amounts. Further research needs to be done with this silicone matrix used in our study to determine repeatability; and to identify the peaks found.

CHAPTER 5

EVALUATION OF CHEMICAL COMPOUNDS FOUND ON HUMAN SKIN AND IN BEAUTY PRODUCTS TO DETERMINE LEVELS OF ATTRACTIVENESS TO *Aedes aegypti* IN A 10 PORT OLFACTOMETER

Introduction

The female mosquito's driving force in life is to reproduce, and to attain this goal, she must seek out blood. However, blood is hidden within animals and the phagostimulant properties found in blood are contained within the red-blood cells. Mosquitoes, therefore, must locate a host using other cues, possibly cues emanating from the skin (Strauss et al. 1968).

Many compounds collected from humans have been found to be attractive such as alanine, lysine, sex hormones and lactic acid (Bar-Zeev et al. 1977). Still other compounds collected from skin samples act synergistically with lactic acid and can be used to develop synthetic attractants that do not require CO_2 (Bernier et al. 2001).

Determining the correct blend of compounds to create lotions and beauty cremes is extremely important for the cosmetic industry. Much of the early work in olfaction was funded by cosmetic companies trying to create beauty products that did not attract flies and biting insects. Many synthetic and natural compounds that are used in beauty products have been studied and identified as either attractant or repellent to some mosquito species (Butler and Katz 1987).

This study was undertaken to determine attractant qualities of certain compounds found either naturally on human skin or in beauty products. Some of these products were chosen because they were determined to be attractants to other blood feeding insects, attractant to *Aedes aegypti*, or commonly found on skin or in beauty products.

Methods and Materials

Mosquitoes Used in Trial

Mosquitoes used were handled with the same techniques as described in Chapter Two.

Colony

Mosquitoes were kept in colony as described in Chapter Two.

Olfactometer

The olfactometer used is described in the materials and methods of Chapter Two.

Artificial Host

The standard (3776) artificial host medium was used in this trial with the same methods of treating the membrane as described in Chapter Two.

In most trials, nine of the ten, "host" membranes were treated with test chemicals, while the tenth membrane served as a control. Some trials were also run by treating the air source instead of the membrane. This was accomplished by inserting a plastic vial containing treatments plus blotter paper (Figure 5-1) into the air stream. Other trials consisted of a mixture of air stream or membrane treatment.

Data Recording

As outlined in Chapter Two.

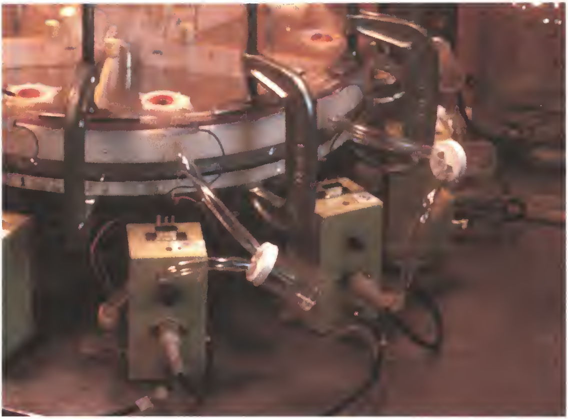


Figure 5-1. Olfactometer set up with air treatments placed in snap cap vials.

Procedure

This study consisted of four trials, each of which were composed of five to ten eight-hour replications (individual tests run once or twice a day, during at least one prime feeding time, comparing subjects). Replications were used in the final statistical analysis if they had no mechanical, electrical or behavioral complications, such as amplifiers not communicating with the computer or sensors shorting, insects trapped behind sensors or no activity recorded for the standard, during a replication. Also, replications that averaged not more or less than 50% different than the trial average were used. Replications with a low average activity implied poor mosquito quality and replications with a high average activity implied insects were trapped underneath sensors, in effect completing the circuit continuously.

Trial 1. In this test various chemicals were compared versus a standard control (3776- blood, agar, and BSS® Plus), an attractant control, and a repellent control (Table 5-1). All treatments were tested in the air stream. Some chemicals were liquid and some were powdered, while lanolin was wax-like. To control for these differences all chemicals were measured by gram weight, at approximately 0.0025g, and placed with a small piece of blotter paper directly in a clean snap cap vial to be placed in the air stream (Figure 5-1). The one exception was the lanolin, which was heated to its liquid state before being weighed and put in the vial.

Table 5-1. Treatments and amounts used in trial 1of chemical study

Number	Treatment	Amount Used
3776	Blood, Agar and BSS® Plus	n/a
3906	OFF*	0.0025 g
3928	Musk 781**	0.0025 g
4783	Progesterone	0.0025 g
4784	Uric Acid	0.0025 g
4785	Squalene	0.0025 g
4786	Lanolin	0.0025 g
4787	Guanine	0.0025 g
4791	Estrone	0.0025 g
4792	Xanthine	0.0025 g

*known repellent
**known attractant

Trial 2. In this test various chemicals were compared with a standard control (3777-agar and BSS® I), an attractant control, and two repellent controls (Table 5-2). The artificial medium was changed to determine if the blood and attractants (BSS® II) used in trial 1 were masking any chemical activity (Braverman et al. 1991). For this trial, liquid chemicals were placed on the surface of the artificial host, while powdered chemicals were placed in the air stream (Table 5-2). Again, approximately 0.0025 g of each chemical was used.

Table 5-2. Treatments, placement and amounts used in trial 2 of chemical study

Number	Treatment	Amount Used	Placement
3777	Agar and BSS® I	n/a	n/a
3906	OFF*	0.0025 g	Surface
3928	Musk 781**	0.0025 g	Surface
3967	Mosquitosafe*	0.0025 g	Surface
4783	Progesterone	0.0025 g	Air stream
4784	Uric Acid	0.0025 g	Air stream
4785	Squalene	0.0025 g	Surface
4787	Guanine	0.0025 g	Air stream
4791	Estrone	0.0025 g	Air stream
4792	Xanthine	0.0025 g	Air stream

*known repellent

**known attractant

Trial 3. In this test various chemicals were compared with a standard control (3778- agar and BSS® Plus), an attractant control, and two repellent controls (Table 5-3). The artificial medium was changed to increase activity by the addition of BSS® II, while still controlling for any masking effects that blood may exert on the chemicals tested (Braverman et al. 1991). Once again, for this trial, liquid chemicals were placed on the surface of the artificial host, while powdered chemicals were placed in the air stream (Table 5-3), and approximately 0.0025 g of each chemical was used.

Table 5-3. Treatments, placement and amounts used in trial 3 of chemical study

Number	Treatment	Amount Used	Placement
3778	Agar and BSS® Plus	n/a	n/a
3906	OFF*	0.0025 g	Surface
3928	Musk 781**	0.0025 g	Surface
3967	Mosquitosafe*	0.0025 g	Surface
4780	Cholesterol @ 1mg/ml***	0.0025 g	Air stream
4783	Progesterone	0.0025 g	Air stream
4785	Squalene	0.0025 g	Surface
4787	Guanine	0.0025 g	Air stream
4791	Estrone	0.0025 g	Air stream
4792	Xanthine	0.0025 g	Air stream

*known repellent

**known attractant

*** dissolved in acetone

Trial 4. This trial was different in that it was a *t*-test which only compared one chemical to a standard (3778- agar and BSS® Plus) (Table 5-4). The normal set up allows 10 choices, but for this trial, the olfactometer was divided into individual two choice chambers, with five replications running at the same time. Treatment was in the air stream (Figure 5-2).

Table 5-4. Treatments used in trial 4 of chemical study

Number	Treatment	Amount Used
3778	Agar and BSS® Plus	0.0025 g
4792	Xanthine	0.0025 g

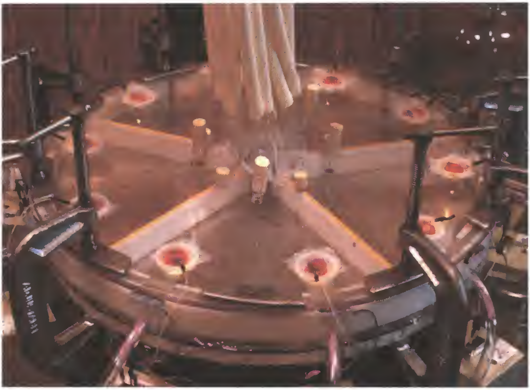


Figure 5-2. Olfactometer set up for trial 4. Two-choice pie design with five replications being run concurrently.

Statistical Analysis

The test was designed as a randomized multi-choice test, as described in Chapter Two. For trial 4, a paired *t*-test was run using two randomly placed artificial hosts. Normalized data were compared to the standard using a one-tailed *t*-test, to determine whether there was any significant difference between samples, for all trials while

ANOVA was used only on trials that allowed 10-choices. Data were normalized as detailed in Chapter Two.

Results and Discussion

Trial 1

Ten repetitions were recorded for trial 1. Attractiveness was measured in average bite-seconds per repetition. Figure 5-3 shows the normalized data with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 5-5).

Table 5-5. Analysis of variance for trial 1 of chemical study, to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	9222.98	9	1024.78	17.55	<0.0001	1.89
Treatment	2503.96	9	278.22	4.77	<0.0001	1.89
Interaction	39113.19	81	482.88	8.27	<0.0001	1.29
Error	40866.35	700	58.38			
Total	91706.48	799				

In this trial, only one chemical treatment, 4786- lanolin, showed any significant difference ($\alpha = 0.05, p = 0.04$) from 3776 (Table 5-6). Lanolin was significantly less attractive than the untreated membrane. Lanolin is a compound (wool fat) that is found on sheep skin and is used in many beauty products (Poucher 1991). Some researchers have speculated that lanolin is a natural defensive mechanism along with the wool itself that helps to protect sheep from insects. Lanolin is a waxy substance which as a surface treatment may be a physical barrier as much as, if not more than, a chemical barrier (Braverman et al. 1991). More research would need to be done to determine if the repellency found in this study could be repeated with air stream treatments as well as surface treatments.

Table 5-6. Standard two-sample *t*-test for trial 1 of chemical study, assuming unequal variances comparing bite-second data for all treatments

	3776	3906	3776	3928	3776	4783	3776	4784	3776	4785
Mean	53.98	47.03	53.98	49.65	53.98	45.89	53.98	40.57	53.98	50.03
Variance	439.33	168.26	439.33	315.67	439.33	483.95	439.33	910.77	439.33	207.49
P(T<=t) one-tail	0.19		0.31		0.21		0.13		0.32	

	3776	4786	3776	4787	3776	4791	3776	4792
Mean	53.98	36.59	53.98	49.71	53.98	51.93	53.98	46.92
Variance	439.33	414.41	439.33	1271.17	439.33	710.00	439.33	457.06
P(T<=t) one-tail	**0.04**		0.37		0.43		0.23	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.10$.

***Where 3776 is the untreated standard with medium containing blood, agar and BSS® Plus.

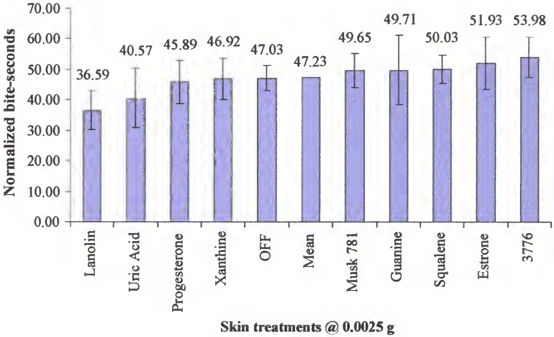


Figure 5-3. Normalized data graph for chemical attractancy trial 1, showing the average bite-seconds (for ten repetitions) plus the standard error. Where 3776 is the untreated standard medium containing blood, agar and BSS® Plus.

Trial 2

Ten repetitions were recorded for trial 2. Attractiveness was measured in average bite-seconds per repetition. Figure 5-4 shows the normalized data with the average bite-seconds for each treatment and the overall mean. All treatments were significantly different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 5-7).

In this trial, six of nine chemical treatments were significantly different from 3777. Treatments 3906 (OFF), 3928 (Musk 781), and 3967 (Mosquitosafe) were significantly less attractive than 3777 ($\alpha = 0.01, p = 0.003, \alpha = 0.10, p = 0.08, \alpha = 0.001, p = 0.0001$) and treatments 4787 (guanine), 4791(estrone), and 4792 (xanthine) were significantly more attractive than 3777 ($\alpha = 0.10, p = 0.07, \alpha = 0.10, p = 0.08, \alpha = 0.05, p = 0.02$) (Table 5-8).

Table 5-7. Analysis of variance for trial 2 of chemical study, to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	2201.52	9	244.61	8.51	<0.0001	1.89
Treatment	25906.19	9	2878.47	100.20	<0.0001	1.89
Interaction	36584.14	81	451.66	15.72	<0.0001	1.29
Error	20109.41	700	28.73			
Total	84801.26	799				

Table 5-8. Standard two-sample *t*-test for trial 2 of chemical study, assuming unequal variances comparing bite-second data for all treatments

	3776	3906	3776	3928	3776	3967	3776	4783	3776	4784
Mean	35.43	14.54	35.43	26.22	35.43	3.18	35.43	37.79	35.43	48.15
Variance	300.22	107.13	300.22	83.86	300.22	9.86	300.22	295.87	300.22	892.6
P(T<=t) one-tail	**0.003**		**0.08**		**0.0001**		0.38		0.13	

	3776	4785	3776	4787	3776	4791	3776	4792
Mean	35.43	27.16	35.43	48.16	35.43	52.59	35.43	59.93
Variance	300.22	447.10	300.22	343.89	300.22	1050.54	300.22	815.37
P(T<=t) one-tail	0.18		**0.07**		**0.08**		**0.02**	

*Significant comparisons are bold.

**P-values are significant at α= 0.10, 0.01, 0.001.

***Where 3777 is the untreated standard medium containing agar and BSS® I.

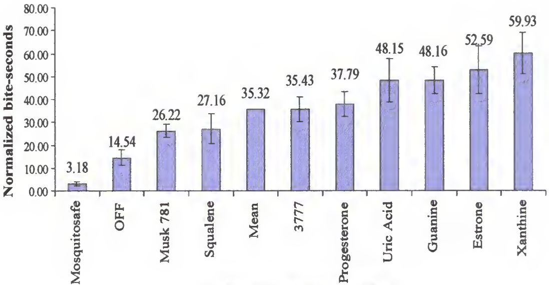


Figure 5-4. Normalized data graph for chemical attractancy trial 2, showing the average bite-seconds (for ten repetitions) plus the standard error. Where 3777 is the untreated standard medium containing agar and BSS® I.

This trial used an artificial host medium that was primarily just a buffer solution. It was determined in Chapter Two that this artificial medium is not very attractive to mosquitoes. However, in this study two known repellents (OFF and MosquitoSafe) as well as a compound that is used by the UF Medical and Veterinary Research lab as a mosquito attractant, Musk 781 (International Flavors and Fragrances) was found to be significantly less attractive than the untreated standard media. This switch in attractancy for Musk 781, could be due to inhibition in the presence of other compounds or possibly the dose used was too low to stimulate attraction. Another possibility is that this compound's attraction may be based upon a chemical reaction that occurs only in the presence of blood and/or non-blood attractants (BSS® Plus). More research needs to be done to determine the exact specifications of the attractant properties of this chemical. Three other compounds were found to be more attractive than the untreated medium standard (3777). This was expected, since the base host medium contained no attractants. One of the compounds, guanine, is a proven tick assembly pheromone (Otieno et al. 1985). Another one of the compounds, estrone, has been shown to be an attractant for

Aedes aegypti when small amounts were volatilized in an olfactometer (Brown 1966). Here larger amounts in the air stream also seem to be an attractant. The third compound, xanthine, is a by-product of ATP dephosphorylation and has been defined as non-stimulatory (Galun 1987). However, in the presence of an unattractive base medium and as an air stream treatment, it appears to have some stimulatory abilities.

Trial 3

Seven repetitions were recorded for trial 3. Attractiveness was measured in average bite-seconds per repetition. Figure 5-5 shows the normalized data with the average bite-seconds for each treatment and the overall mean. All treatments were different ($\alpha = 0.10, p < 0.0001$) and the interaction between each treatment was significant ($\alpha = 0.10, p < 0.0001$), (Table 5-9).

In this trial, two chemical treatments were significantly different from 3778. Treatments 3906 (OFF) and 3967 (MosquitoSafe) were significantly less attractive than 3778 ($\alpha = 0.05, p = 0.02, p = 0.01$), (Table 5-10).

Table 5-9. Analysis of variance for trial 3 of chemical study to determine differences between treatments

Source of Variation	SS	df	MS	F	P-value	F crit
Replication	5196.35	6	866.06	26.38	<0.0001	2.12
Treatment	16174.54	9	1797.17	54.74	<0.0001	1.90
Interaction	41545.71	54	769.36	23.43	<0.0001	1.36
Error	16087.06	490	32.83			
Total	79003.66	559				

Table 5-10. Standard two-sample t-test for trial 3 of chemical study, assuming unequal variances comparing bite-second data for all treatments

	3778	3906	3778	3928	3778	3967	3778	4780	3778	4783
Mean	55.24	22.84	55.24	50.17	55.24	17.82	55.24	59.14	55.24	57.84
Variance	1051.16	138.06	1051.16	872.70	1051.16	234.76	1051.16	1679.20	1051.16	586.31
P(T<=t) one-tail	**0.02**		0.38		**0.01**		0.42		0.43	

	3778	4785	3778	4787	3778	4791	3778	4792
Mean	55.24	50.83	55.24	63.35	55.24	42.40	55.24	64.46
Variance	1051.16	820.51	1051.16	581.71	1051.16	903.99	1051.16	708.94
P(T<=t) one-tail	0.40		0.30		0.23		0.29	

*Significant comparisons are bold.

**P-values are significant at α = 0.05.

***Where 3778 is the untreated standard medium containing agar and BSS® Plus.

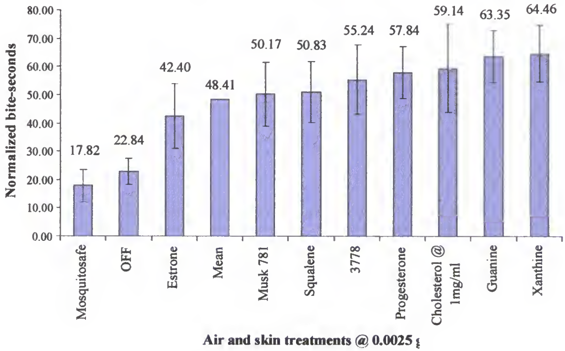


Figure 5-5. Normalized data graph for chemical attractancy trial 3, showing the average bite-seconds (for seven repetitions) plus the standard error. Where 3778 is the untreated standard medium containing agar and BSS® Plus.

After the addition of BSS® II to the standard artificial medium for this trial, the activity increased. The normalized bite-seconds for the untreated standard almost doubled when compared to trial 2, which used standard 3777 (agar and BSS® I). With more attraction being attained by the untreated host, less significance was seen for the other chemicals. The known repellents remained repellent, but no other chemical was significantly different than the untreated host. For this trial, the known attractant (Musk 781) was not significantly different than the untreated host. This could be due to a dosage problem, or interaction with neighboring compounds. Or as previously stated, it's "known" attraction might be based on an interaction with the components of the host medium, with those components changed, it's attraction levels change. Follow up studies need to be pursued.

Trial 4

Five repetitions were recorded for trial 4. Attractiveness was measured in average bite-seconds per repetition. Figure 5-6 shows the normalized data with the average bite-seconds for each treatment and the overall mean.

In this trial, treatment 4792 (xanthine) was significantly less attractive than 3778 ($\alpha = 0.05$, $p = 0.02$), (Table 5-11).

Table 5-11. Standard two-sample t-test for trial 4 of chemical study, assuming unequal variances comparing bite-second data for both treatments

	3778	4792
Mean	54.82	22.72
Variance	481.38	458.90
P(T<=t) one-tail	**0.02**	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.05$.

***Where 3778 is the untreated standard medium containing agar and BSS® Plus.

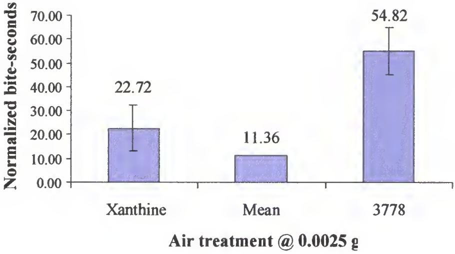


Figure 5-6. Normalized data graph for chemical attractancy trial 4, showing the average bite-seconds (for five repetitions) plus the standard error. Where 3778 is the untreated standard medium containing agar and BSS® Plus.

This trial was done as a follow-up to trials 1, 2 and 3. In trial 1, xanthine was not significantly different than the untreated host medium, 3776 (blood, agar and BSS® Plus), although it had a lower number of bite-seconds recorded. In trial 2, xanthine was found to be significantly more attractive than the untreated host medium, 3777 (agar and BSS® I). In trial 3, it was found to be statistically the same as the untreated host medium, 3778 (agar and BSS® Plus), but with a higher number of bite-seconds recorded. By testing xanthine in a paired T set up against only the untreated standard, 3778, possible interactions from extraneous sources could be removed. Our results suggest that xanthine's demeanor is non-stimulatory in nature (Galun 1987), but that the presence of blood in the medium definitely has an effect on the level of attraction of this particular chemical, and possibly others. More research needs to be done with chemicals that are commonly found on skin or in beauty products to determine chemical reactions with blood or to determine levels of attraction or repellency.

CHAPTER 6

EVALUATION OF SYNTHETIC CHOLESTEROL AS AN ATTRACTANT FOR *Aedes aegypti* IN A 10 PORT OLFACTOMETER

Introduction

Mosquitoes require cholesterol from their diet in order to develop eggs (Nation 2002). Therefore, it is something that they must be able to sense with chemoreceptors, possibly located on the antenna or palpi (Butler and Okine 1995).

Everyone has cholesterol on the skin surface, it is generated de novo in cells of the stratum corneum (Wertz 2000). The stratum corneum is the skin's outside layer and is essential in protection of the system from water loss and foreign agents that could be toxic (Caspers et al. 2001). Cholesterol is the most common single lipid found in the stratum corneum, being found at saturating levels (Wertz 2000). Because of the sheer abundance of cholesterol in the skin, it is likely that some may be sensed by mosquitoes.

There are many chemicals found in human skin extracts that have not been identified or qualified at this time as attractant or repellent (Bernier et al. 1999).

Anecdotal evidence as well as comparisons of human subjects who have been tested prior to and post anti-cholesterol medications suggest a possible role of cholesterol or one of the many compounds for which cholesterol is a precursor in mosquito attraction (Lehninger et al. 1993). This study will attempt to determine if synthetic cholesterol is an attractant for mosquitoes and if so, at what dose and treatment method.

Methods and Materials

Mosquitoes Used in Trial

Mosquitoes used were handled with the same techniques as described in Chapter Two.

Colony

Mosquitoes were kept in colony as described in Chapter Two.

Olfactometer

The olfactometer used is described in the materials and methods of Chapter Two.

Artificial Host

The standard (3776) artificial host medium was used in this trial with the same methods of treating the membrane as described in Chapter Two and Chapter Two.

Data Recording

As outlined in Chapter Two.

Procedure

This study consisted of six trials, all of which consisted of between two and five eight-hour replications (individual tests that were run once or twice a day, during at least one prime feeding time, comparing subjects). Replications were used in the final statistical analysis as explained in Chapter Two.

For trials 1 and 3, the olfactometer array was modified from a 10-choice setup to a two choice set up or paired *t*-test. It was divided into five separately sealed sections with 32 mosquitoes used in each section. Mosquitoes had a choice of feeding on either a treated or non-treated sample (Figure 6-1).

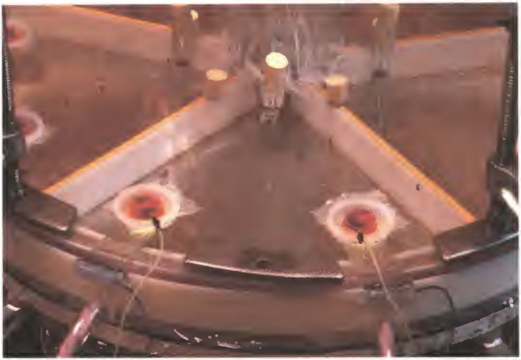


Figure 6-1. Two-choice design, giving mosquitoes the opportunity to feed on either a treated or non-treated membrane or air-stream.

For trial 4, the olfactometer array was modified from a 10-choice set up to a four choice set up.

For trials 5 and 6, the olfactometer was used with the standard 10-choice set up. A dosage curve was tested using dosages varying from 0.0001 mg to 0.01 mg of cholesterol (randomly placed within the olfactometer).

Trial 1. This was a two-choice, five repetition trial which utilized air-treatments. Cholesterol was weighed (approximately 0.005 g) into snap cap vials that were placed into the air-stream of the olfactometer (Figure 5-1) and compared to the standard 3776 artificial medium containing blood, agar and BSS® Plus.

Trial 2. This was a repeat of trial 1, with the only difference being the way the air-stream was treated. For this trial, the 0.005 g of cholesterol was weighed onto a small piece of lens paper and then folded, stapled and inserted directly into the air-stream tube (Figure 6-2).

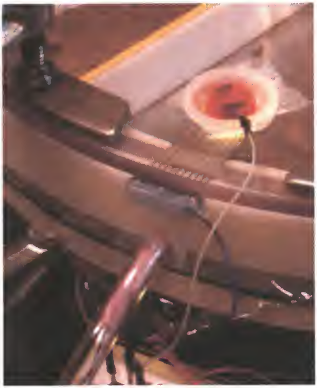


Figure 6-2. Paper packets treated and inserted inside air tubing for air treatment in Trial 2.

Trial 3. This was a two-choice, five repetition trial which utilized membrane treatments. Approximately 0.0025 g of powdered cholesterol was placed on each membrane and compared in the paired *t*-test design with standard 3776 artificial medium containing blood, agar and BSS® Plus.

Trial 4. This was a four-choice, two repetition trial which utilized air-stream treatments. Each repetition had a choice of one dose of cholesterol (0.005 g) free powder placed in snap cap vials in the air-stream versus three standards, 3776 artificial medium containing blood, agar and BSS® Plus. Treated sample was arranged on the end next to the side of the separation, with three untreated samples in the rest of the openings. This arrangement was used to determine if treatment had any affect on its neighbors.

Trial 5. This was a ten-choice, five repetition trial which utilized air-stream treatments. Each repetition compared four doses of cholesterol (0.001 g, 0.0025 g, 0.005 g, and 0.01 g) with five standards (3776- artificial medium containing blood, agar and BSS® Plus) and one standard repellent (3906- OFF), at 0.0025 g. Each treatment was paired with an untreated standard and then randomly placed within the olfactometer for each repetition.

Trial 6. This was a ten choice, five repetition trial which utilized membrane treatment. Each repetition compared nine doses of cholesterol (0.0001 mg, 0.002 mg, 0.0003 mg, 0.0004 mg, 0.0006 mg, 0.0007 mg, 0.0008 mg, 0.0009 mg and 0.01 mg) with one standard (3776- artificial medium containing blood, agar and BSS® Plus). Cholesterol doses as well as untreated standard were randomly placed in the olfactometer prior to each repetition.

Chemical Preparation. For all trials except trial 6, synthetic powdered cholesterol (purchased from Acros Organics) was used. It was weighed with a Mettler Toledo Precision balance into snap cap vials, paper packets or directly onto the artificial host membrane. For trial 6, the cholesterol was dissolved in an acetone solvent and then measured by volume with an Eppendorf pipetter. Each sample was prepared by using standard dilutions (Table 6-1).

Table 6-1. Dilution chart for Cholesterol doses used in Trial 6

Vial 1	0.5	g of chol	=	100	mg/ml	=	1	mg/10ul
	5	ml of acetone						
Vial 2	0.1	ml of vial 1	=	10	mg/ml	=	0.1	mg/10ul
	0.9	ml of acetone						
Vial 3	0.005	g of chol	=	1	mg/ml	=	0.01	mg/10ul
	5	ml of acetone						
Vial 4	0.9	ml of vial 3	=	0.09	mg/ml	=	0.0009	mg/10ul
	0.1	ml of acetone						
Vial 5	0.8	ml of vial 3	=	0.08	mg/ml	=	0.0008	mg/10ul
	0.2	ml of acetone						
Vial 6	0.7	ml of vial 3	=	0.07	mg/ml	=	0.0007	mg/10ul
	0.3	ml of acetone						
Vial 7	0.6	ml of vial 3	=	0.06	mg/ml	=	0.0006	mg/10ul
	0.4	ml of acetone						
Vial 8	0.5	ml of vial 3	=	0.05	mg/ml	=	0.0005	mg/10ul
	0.5	ml of acetone						
Vial 9	0.4	ml of vial 3	=	0.04	mg/ml	=	0.0004	mg/10ul
	0.6	ml of acetone						
Vial 10	0.3	ml of vial 3	=	0.03	mg/ml	=	0.0003	mg/10ul
	0.7	ml of acetone						
Vial 11	0.2	ml of vial 3	=	0.02	mg/ml	=	0.0002	mg/10ul
	0.8	ml of acetone						
Vial 12	0.1	ml of vial 3	=	0.01	mg/ml	=	0.0001	mg/10ul
	0.9	ml of acetone						

*Vials three through twelve were used for Trial.

Statistical Analysis

The test was designed as a randomized multi-choice test, as described in Chapter 2. For trials 1, 2 and 3 a paired *t*-test was run using two randomly placed artificial hosts. Normalized data were compared as described in Chapters Two and Five.

Results and Discussion

Trial 1

Five repetitions were recorded for trial 1. Data comparing 0.005 g of cholesterol in the air stream versus the untreated standard 3776 artificial medium containing blood, agar and BSS$^{®}$ Plus were normalized using the equation: SQRT of n + 1 (Table 6-2). Attractiveness was measured in average bite-seconds per repetition. Cholesterol was significantly more attractive than the standard ($\alpha = 0.10, p = 0.09$) (Table 6-3). Figure 6-3 shows the normalized data with the average bite-seconds for each treatment per repetition and the overall average.

Table 6-2. Normalized bite-second data for trial 1 of cholesterol study

Rep #	3776	Cholesterol
1	13.05	7.86
2	33.35	45.37
3	16.09	55.40
4	26.69	68.15
5	35.80	33.37

*Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-3. Standard two-sample *t*-test for trial 1 of cholesterol study, assuming unequal variances comparing bite-second data for both treatments

	3776	Cholesterol
Mean	24.99	42.03
Variance	102.88	528.65
P(T<=t) one-tail	**0.09**	

*Significant comparisons are bold.
**P-values are significant at $\alpha = 0.10$.
***Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

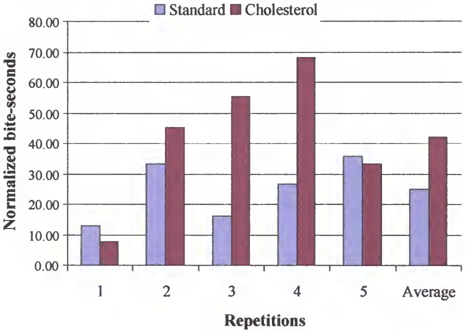


Figure 6-3. Normalized bite-second data for trial 1 of cholesterol study. Standard versus cholesterol air treatment. Where the standard is an artificial medium containing blood, agar and BSS® Plus.

Cholesterol was placed in the air stream at a dosage of 0.005 g. This dosage was based upon previous chemical attractant trials for air stream treatment. Generally air stream treatments are double the rate of surface or membrane treatments (Butler and Katz 1987). As an air stream treatment, cholesterol at a much higher rate than what is normally found on skin does appear to have attractant qualities (Di Nardo et al. 1998).

Trial 2

Five repetitions were recorded for trial 2. Data comparing 0.005 g of cholesterol in the air stream versus the untreated standard 3776 artificial medium containing blood, agar and BSS® Plus were normalized using the equation: SQRT of n + 1 (Table 6-4). Attractiveness was measured in average bite-seconds per repetition. There was no significant difference between the standard 3776 artificial medium containing blood, agar and BSS® Plus and cholesterol (Table 6-5). Figure 6-4 shows the normalized data with the average bite-seconds for each treatment per repetition and the overall average.

Table 6-4. Normalized bite-second data for trial 2 of cholesterol study

Rep #	3776	Cholesterol
1	46.45	57.68
2	47.93	46.91
3	43.12	27.09
4	55.21	77.42
5	71.13	42.08

*Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-5. Standard two-sample *t*-test for trial 2 of cholesterol study, assuming unequal variances comparing bite-second data for both treatments.

	3776	Cholesterol
Mean	52.77	50.24
Variance	124.92	351.92
P(T<=t) one-tail	0.40	

*P-values are significant at $\alpha = 0.10$.

**Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

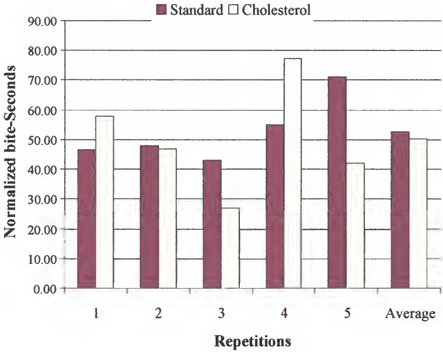


Figure 6-4. Normalized data for trial 2 of cholesterol study. Standard versus cholesterol air treatment. Where the standard is an artificial medium containing blood, agar and BSS® Plus.

All aspects of this trial were the same as trial 1, except for the method of air stream treatment. In this trial, instead of using snap cap vials, a paper packet was created and inserted directly into the air tube below each artificial host. In this trial, there was no significant difference between the two treatments, we can assume that the method of delivery was faulty.

Trial 3

Five repetitions were recorded for trial 3. Data comparing 0.0025 g of cholesterol on the membrane versus the untreated standard 3776 artificial medium containing blood, agar and BSS® Plus were normalized using the equation: SQRT of n + 1 (Table 6-6). Attractiveness was measured in average bite-seconds per repetition. There was no significant difference between the standard, 3776 artificial medium containing blood, agar and BSS® Plus and cholesterol (Table 6-7). Figure 6-5 shows the normalized data with the average bite-seconds for each treatment per repetition and the overall average.

Table 6-6. Normalized bite-second data for trial 3 of cholesterol study

Rep #	3776	Cholesterol
1	32.99	65.26
2	34.28	77.68
3	50.75	19.47
4	39.41	66.11
5	54.81	36.93

*Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-7. Standard two-sample *t*-test for trial 3 of cholesterol study, assuming unequal variances comparing bite-second data for both treatments

	3776	Cholesterol
Mean	42.45	53.09
Variance	96.80	578.49
P(T<=t) one-tail	0.20	

*P-values are significant at $\alpha = 0.10$.

**Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

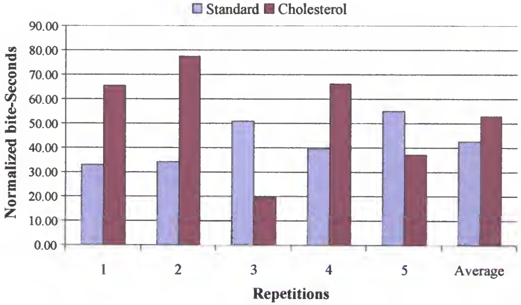


Figure 6-5. Normalized data for trial 3 of cholesterol study. Standard versus cholesterol skin treatment. Where the standard is an artificial medium containing blood, agar and BSS® Plus.

For this trial, the powdered cholesterol (Acros) was weighed directly onto the membrane surface, at half of the dosage used for air stream treatments (Butler and Katz 1987). There was no significant difference between treatments. Possibly, the powder created a physical barrier that impeded the feeding attempts of the mosquitoes or as a surface treatment was not properly retained or the dosage was too high. There were some problems with the powdered cholesterol on the surface of the membrane and artificial host. The powder weight would change rapidly and excess could not be easily removed from sample if overweight. Mosquitoes could also easily move powder around by tarsal contamination, thereby neutralizing treatments.

Trial 4

Two repetitions were recorded for trial 4. Data were normalized using the equation: SQRT of N + 1 (Table 6-8). Attractiveness was measured in average bite-seconds per repetition. Cholesterol was significantly less attractive than the standard (Table 6-9). Figure 6-6 shows the normalized data with the average bite-seconds for each treatment per repetition and the overall average. Normalized data from all standards (3776A-C) for both reps versus cholesterol sample are shown in Table 6-10. Standard 3776B was significantly more attractive than standard 3776C ($\alpha = 0.10, p = 0.06$) (Table 6-11). This suggests that position two was positively affected by being one treatment away from the treated sample. Had cholesterol been significantly more attractive than the non-treated standards this may have held true, however, since the mean cholesterol bite-seconds were significantly less attractive than the overall mean standard bite-seconds, no generalized statement can be made about position from treatment.

Table 6-8. Normalized mean bite-second data for trial 4 of cholesterol study

Rep #	Mean 3776*	Cholesterol
1	69.45	33.70
2	61.39	33.63

* Each repetition contained 3 standards that were averaged to get the mean standard.

**Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-9. Standard two-sample *t*-test for the mean of trial 4 of cholesterol study, assuming unequal variances comparing mean bite-second data for both treatments.

	Mean 3776	Cholesterol
Mean	65.42	33.67
Variance	32.50	0.00
P(T<=t) one-tail	**0.04**	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.10$.

***Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

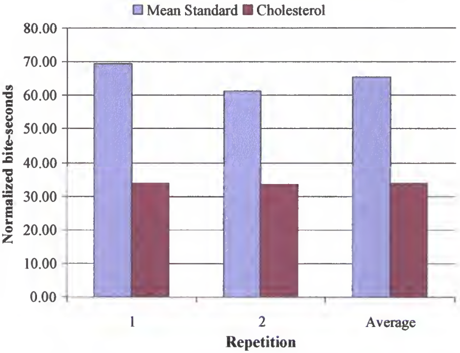


Figure 6-6. Normalized data for trial 4 of cholesterol study. Mean standard versus cholesterol air treatment. Where the mean standard is an artificial medium containing blood, agar and BSS® Plus.

Table 6-10. Normalized bite-second data for trial 4 of cholesterol study. Treated sample versus 3776 artificial medium containing blood, agar and BSS® Plus.

	Cholesterol	3776A	3776B	3776C
Rep 1	33.70	81.80	69.06	54.83
Rep 2	33.63	43.82	87.82	40.62

*Letter designations determine position from treated sample, as shown.

Table 6-11. Standard two-sample *t*-test for trial 4 of cholesterol study, assuming unequal variances comparing bite-second data for all Standards.

	3776A	3776B	3776A	3776C	3776B	3776C
Mean	62.81	78.44	62.81	47.73	78.44	47.73
Variance	721.05	176.08	721.05	100.99	176.08	100.99
P(T<=t) one-tail	0.30		0.30		**0.06**	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.10$.

***Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

****Letter designations determine position from treated sample.

For this study, the same dose of cholesterol was used in the air stream as trial 1. In trial 1, cholesterol was significantly more attractive than the untreated standard, whereas in this trial it was significantly less attractive than the untreated standard. The possibility exists that because of the addition of extra untreated hosts, which were loaded with blood and non-blood attractants (Galun et al. 1993), that attractant qualities of the cholesterol may have been masked (Braverman et al. 1991).

Trial 5

Five repetitions were recorded for trial 5. Data comparing dosages of cholesterol with 3776, the standard artificial medium containing blood, agar and BSS® Plus were normalized using the equation: SQRT of n + 1 (Table 6-12). Attractiveness was measured in average bite-seconds per repetition. Cholesterol dosage, 0.0025 g was significantly less attractive than the standard, 3776 (containing blood, agar and BSS® Plus) ($\alpha = 0.10, p = 0.07$). The standard repellent, OFF, was significantly less attractive than the standard, 3776 (containing blood, agar and BSS® Plus) and cholesterol dosages, 0.001 g and 0.01 g. ($\alpha = 0.10, p = 0.04, 0.04, 0.05$) (Table 6-13). Figure 6-7 shows the normalized data with the average bite-seconds for each treatment versus the overall average.

Table 6-12. Normalized bite-second data for trial 5 of cholesterol study

	Cholesterol Dose (grams)						
Rep #	3776	0.001	0.0025	0.005	0.01	OFF - 0.0025	Average
1	57.64	51.44	61.45	55.19	69.16	65.10	60.30
2	56.26	60.12	1.32	34.18	48.00	1.00	41.54
3	44.04	48.78	56.07	21.11	73.38	22.70	48.05
4	54.62	48.90	14.37	32.21	21.88	1.00	34.56
5	75.93	70.55	42.47	78.43	109.44	46.83	74.06

*Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-13. Standard two-sample *t*-test for trial 5 of cholesterol study, assuming unequal variances comparing bite-second data for all treatments

	3776	0.001	3776	0.0025	3776	0.005	3776	0.01
Mean	57.70	55.96	57.70	35.14	57.70	44.22	57.70	64.37
Variance	132.68	88.00	132.68	689.79	132.68	517.49	132.68	1052.13
P(T<=t) one-tail	0.40		**0.07**		0.14		0.34	

	3776	OFF - 0.0025	OFF - 0.0025	0.001	OFF - 0.0025	0.0025	OFF - 0.0025	0.005
Mean	57.70	27.33	27.33	55.96	27.33	35.14	27.33	44.22
Variance	132.68	803.70	803.70	88.00	803.70	689.79	803.70	517.49
P(T<=t) one-tail	**0.04**		**0.04**		0.33		0.16	

	OFF - 0.0025	0.01
Mean	27.33	64.37
Variance	803.70	1052.13
P(T<=t) one-tail	**0.05**	

*Significant comparisons are bold.

**P-values are significant at $\alpha = 0.10$.

***Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

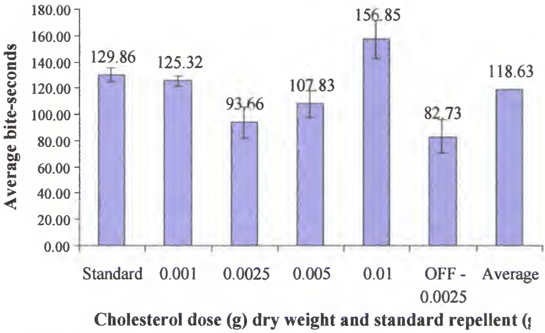


Figure 6-7. Normalized air treatment data for trial 5 of cholesterol study. Standard versus four cholesterol dosages plus the overall average, including standard error. Where the standard is an artificial medium containing blood, agar and BSS® Plus.

In this trial, the 0.0025 g dose of cholesterol in the air stream was significantly less attractive than the standard, 3776 (containing blood, agar and BSS® Plus) as was the standard repellent, OFF. Since OFF was repellent in this study, as was predicted, it can be assumed that mosquito activity was normal. Once again, the dosage of cholesterol that had previously shown to be attractive in the air stream, was not significantly different than the standard, 3776 (containing blood, agar and BSS® Plus) for this trial. Possibilities for these results include masking by the addition of other dosages (Braverman et al. 1991) or sheer overloading of the insect's chemoreceptors, inadvertantly causing inhibition (Hoffman and Miller 2002) by having so many air stream treatments of the same chemical, with only one true repellent in the trial.

Trial 6

Five repetitions were recorded for trial 6. Data comparing nine doses of cholesterol with 3776, the standard artificial medium containing blood, agar and BSS®

Plus and 10 µL of acetone on membrane surface were normalized using the equation: SQRT of n + 1 (Table 6-14). Attractiveness was measured in average bite-seconds per repetition. Cholesterol dosages, 0.003 and 0.0007 g were significantly more attractive than the standard, 3776 (containing blood, agar and BSS® Plus and 10µL of acetone on membrane surface) ($\alpha = 0.10, p = 0.08, p = 0.08$) (Table 6-15). Figure 6-8 shows the normalized data with the average bite-seconds for each treatment versus the overall average.

Table 6-14. Normalized bite-second data for trial 6 of cholesterol study

		Cholesterol Dosage (mg)								
Rep #	3776	0.0001	0.0002	0.0003	0.0004	0.0006	0.0007	0.0008	0.0009	0.01
1	40.66	19.83	54.49	44.93	51.51	56.07	63.65	80.28	28.47	46.02
2	36.03	78.68	24.13	46.65	35.13	25.33	43.49	67.61	13.00	13.08
3	7.98	17.00	10.40	48.23	17.43	10.73	24.98	3.73	37.82	60.43
4	50.69	79.31	75.07	56.88	10.24	84.66	86.46	27.19	66.19	82.73
5	22.42	78.30	27.58	29.98	30.42	59.47	41.99	68.72	73.97	37.92

*Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

Table 6-15. Standard two-sample *t*-test for trial 6 of cholesterol study, assuming unequal variances comparing bite-second data for 2 cholesterol dosages and the standard

	3776	0.0003	3776	0.0007
Mean	31.56	45.33	31.56	52.11
Variance	277.15	94.82	277.15	556.43
P(T<=t) one-tail	0.08		0.08	

*Significant comparisons are shown.

**P-values are significant at $\alpha = 0.10$.

***Where 3776 is an artificial medium containing blood, agar and BSS® Plus.

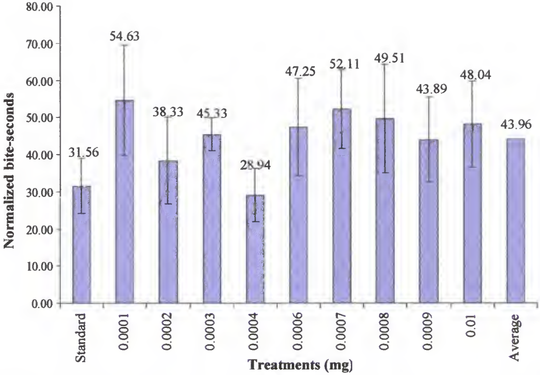


Figure 6-8. Normalized data for five repetitions of trial 6 of cholesterol study. Nine doses of cholesterol versus the standard and overall average, including standard error. Where the standard is an artificial medium containing blood, agar and BSS® Plus.

This trial attempted to combat air stream overload, by treating the membrane or surface of the artificial host. To get a more even distribution and lower dosages, the cholesterol powder was dissolved in acetone. This liquid solution now allowed more control over surface dosing treatments. With this treatment configuration, two doses of cholesterol were found to be attractant. Demonstrating the critical nature of dosage dependency for mosquito response to cholesterol. The doses did not receive the highest bite-seconds, but were lower in variation than the dose with the highest bite-seconds recorded (0.0001 mg). There were still many inconsistencies in the reaction of the mosquitoes to the cholesterol dosages. No accurate dose could be determined from this trial.

CHAPTER 7
DISCUSSION AND FURTHER STUDIES

Mosquitoes have been one of man's worst enemies throughout history. They can cause economic losses in agriculture, as well as much human death in many parts of the world. There are many methods of chemical control of mosquitoes, none of which are entirely effective, including personal and widespread pesticide treatments. This release of harmful chemicals into the environment has spurred researchers for decades to investigate the signals that bring mosquitoes to a potential host (Bernier et al. 2000).

Aedes aegypti is a very important species to the field of Medical Entomology. They transmit deadly diseases in many areas of the world, and they are relatively easy to maintain in colony. These factors allow scientists to do preliminary studies with this species and then, if warranted, design more studies for field trials or other disease carrying species (Adams 1999).

Insects that carry deadly diseases can not be allowed to feed on human subjects during the course of research in the United States. Artificial hosts are very important in the study of insects that transmit disease to humans. With current regulations on human use, it is impossible to feed a mosquito on a human, much less try to perform disease transmission studies with humans. Therefore, having an appropriate membrane feeding system in place is essential. These systems allow mosquitoes and other blood feeding insects to be kept in colonies at research facilities without feeding on live hosts (Bunner et al. 1989). The artificial media used in these systems must be adjusted to fit the needs of the insect and the research. For colony maintenance, a medium must be designed that stimulates the insects to investigate, probe, taste, engorge, and reproduce. This is not required for attraction studies or necessarily for transmission studies. All that is required from these media is that they stimulate the insect to investigate, probe, taste, and possibly try to engorge (Galun 1967, 1987). For our system, a simple bovine blood solution with sodium citrate added on a gauze wrapped cotton ball is all that is necessary to maintain colony production. A mixture of the same blood with agar and BSS® Plus added is used as a medium for the artificial host systems used for attraction trials (Butler et al. 1984). When the components of the medium are investigated separately, what is it that is the most stimulating to *Ae. aegypti*? Do some components mask the test compound? From our study of artificial host media, we have determined that blood is an important stimulant when designing artificial media for attraction trials with CO_2. Although Galun et al. (1985B) state that insects are not chemoreceptive to blood alone, my study shows no difference in media that contain only blood versus media with blood and non-blood attractants; and blood seems to increase stability by probing and membrane contact under the influence of CO_2.

The possibility that components of the artificial media may mask naturally occurring repellents found on skin is daunting. Bernier and Posey (2001) have determined that natural "inhibitors" exist in human extracts, and our results do show naturally "unattractive" subjects, and subjects who become more "unattractive" by the removal of non-blood attractants from the artificial host media. It could be possible that in our zeal to make sure that our artificial host medium is stimulating to the mosquitoes, we have inadvertantly added chemicals that in the presence of CO_2 could mask natural inhibitors. More studies comparing human treated artificial hosts need to be done to determine what the proper ratio of blood and non-blood attractants is for artificial host media.

Much of the research done with *Ae. aegypti*, has helped determine attractant host compounds, some of which have crossed species borders. Initially, mosquitoes are attracted to a potential host by sight and warmth and then the olfactory cues register and the emanating volatiles begin to actually lead the mosquito to her target. Some of these volatiles include L-lactic acid, CO_2, octenol and, others that have yet to be definitively identified (Bernier et al., 1999, 2000, Bernier and Posey 2001, Acree et al. 1968, Braks and Takken 1999).

There are compounds found on human skin or in sweat that cause variations in levels of attraction between human subjects (Shirai et al. 2000). Approximately 275 compounds isolated from human extracts have been identified as attractants and at least one compound has been classified as a mosquito inhibitor (Bernier and Posey 2001). This chemical analysis along with field studies indicating consistent human attractants over long periods of time suggest that there is a possible ratio of compounds present in certain individuals which predispose them to being bitten by mosquitoes (Lindsay et al. 1993).

Identification of individuals as attractant or repellent to mosquitoes and then comparing their individual chemical profiles is the next logical step. Preliminary studies show that there is a difference in these profiles. Some compounds are present in an attractant subject that are not present in a repellent one and vice versa. These unique compounds need to be further identified and tested.

The results of the preferential host study, shows that at least one person in a group of ten significantly stands out as attractant and one significantly stands out as repellent. Many recent studies have shown consistent patterns of attraction for individuals (Ansell et al. 2002, Lindsay et al. 1993, Brady et al. 1997). New research is also now supporting the possibility that some people could be producing inhibitors that make them less attractive or even repellent in comparison to other people (Bernier et al. 2001, McKenzie 2000). Most human secretions are similar in specific compounds, but they tend to vary in amounts of these compounds. This variation is probably what makes some people more attractive than others (Bernier et al. 2000, 2002).

In our study we determined that there were also distinct differences between subjects that had been identified as attractant or repellent by other tests. To follow this study up, more subjects would have to be identified as either attractant or repellent and then tested repeatedly to determine if the differences were consistent or anomalous to our two subjects.

Many cosmetic companies take advantage of research done by entomologists to determine what not to put in their formulations (Butler and Katz 1987). Chemicals are tested and then labeled as attractant or repellent, so that beauty products can be effective and not attractive to insects. Our research on chemicals found in beauty products and on human skin identified lanolin as a repellent. This is a naturally occurring secretion that is commonly used in bath products and lotions. It is also a natural defense of some animals such as sheep against insects (Braverman et al. 1991). My results on all other compounds tested were mixed. For instance, estrone is a known attractant for *Ae. aegypti*, however, only when the artificial host medium was completely lacking attractants, attractant activity was noted for this chemical (Brown 1966). At particular doses some chemicals were attractant and then when re-tested the same chemical were repellent. These skewed results are probably due to lack of knowledge of the proper dosage and presentation for these synthetics. With more testing, it is possible that the appropriate dosage would be found for all of our chemicals, including cholesterol, and then an appropriate designation could be assigned.

Overall, this research has determined that laboratory tests support field findings that some people are more attractive than others to mosquito feeding (Lindsay et al. 1993, Brady et al. 1997), as well as supporting the finding that there is a compound found in human skin secretions that could be an inhibitor of mosquito attraction (Bernier and Posey 2001). Such an inhibitor may be masked by components used in attraction studies. We have also preliminarily determined that humans who are rated as either attractant or repellent have distinctly different chemical profiles, but determinations of the identification of those chemicals was beyond the scope of this research. Synthetic chemicals, which mimic natural body secretions are not as easy to test for attraction as the actual body secretion. Determining actual dosages and levels of each chemical found in human secretions would be extremely helpful in determining the appropriate dosage for synthetic attractants. More research needs to be done to find what aspects of human and mosquito metabolism can be manipulated to better control these insects which cause so much death every year.

APPENDIX A
INFORMED CONSENT TO PARTICIPATE IN RESEARCH

IRB# 210-1998

Informed Consent to Participate in Research

You are being asked to take part in a research study. This form provides you with information about the study. The Principal Investigator (the person in charge of this research) or a representative of the Principal Investigator will also describe this study to you and answer all of your questions. Before you decide whether or not to take part, read the information below and ask questions about anything you do not understand. Your participation is entirely voluntary.

1. Name of Participant ("Study Subject")

____

2. Title of Research Study

Determining factors of preferential host selection by *Aedes aegypti*

3. Principal Investigator and Telephone Number(s)

J. F. Butler:	Home	(352) 378-0603
	Work	(352) 392-1901 ext. 152
Karen McKenzie:	Home	(352) 472-2919
	Work	(352) 392-1901 ext. 169

4. Source of Funding or Other Material Support

University of Florida

5. What is the purpose of this research study?

Currently it is not known why mosquitoes are attracted to some people more than others. The purpose of this study is to determine if the levels of cholesterol and other similar chemicals secreted in human sweat, effect mosquito attraction. Because you have insect attractant characteristics - mosquitoes bite you more than other people around you or they do not seem to bother you as much as the people around you - you are being asked to participate in this research protocol.

6. What will be done if you take part in this research study?

You will be asked to fill out a questionnaire at the beginning of the study, to document any medications you may be taking and also to document some of your daily routines. You will also be asked to wear a small membrane on your forearm for four hours, once a week, for approximately 12 to 15 weeks. The membrane is similar to silicone membranes currently used to treat burn patients and will absorb odors and chemicals that are secreted by the skin. This membrane will be attached to your arm with a nylon hose, similar to panty hose, for a four-hour period. The membrane will be collected by the researchers and placed into a cage with mosquitoes to measure the number of bites that each membrane receives over an eight-hour period. Sweat will periodically be collected during the course of the study. On days that sweat is to be collected, you will be asked to walk around outside for a few minutes and then your sweat will be passively collected. The collected sweat will be analyzed to determine levels of secreted cholesterol and other similar chemicals. Your participation in the study is expected to last no longer than six months.

7. What are the possible discomforts and risks?

There are no known health risks or discomforts that may be associated with participation in this study.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, you may ask questions now or call the Principal Investigator or contact person listed on the front page of this form.

8a. What are the possible benefits to you?

You will not receive any treatment that may potentially benefit you.

8b. What are the possible benefits to others?

If cholesterol is identified as the primary attractant in human sweat, there is potential to develop a more effective mosquito repellent in the future.

9. If you choose to take part in this research study, will it cost you anything?

There are no known financial risks that may be associated with participation in this study.

10. Will you receive compensation for taking part in this research study?

No

11. What if you are injured because of the study?

If you experience an injury that is directly caused by this study, only professional consultative care that you receive at the University of Florida Health Science Center will be provided without charge. However, hospital expenses will have to be paid by you or your insurance provider. No other compensation is offered.

12. What other options or treatments are available if you do not want to be in this study?

Participation in this study is entirely voluntary. You are free to refuse to be in the study, and your refusal will not influence current or future health care you receive at this institution.

13a. Can you withdraw from this research study?

You are free to withdraw your consent and to stop participating in this research study at any time. If you do withdraw your consent, there will be no penalty, and you will not lose any benefits you are entitled to.

If you decide to withdraw your consent to participate in this research study for any reason, you should contact Karen McKenzie or J.F. Butler at (352) 392-1901 ext 169 .

If you have any questions regarding your rights as a research subject, you may phone the Institutional Review Board (IRB) office at (352) 846-1494.

13b. If you withdraw, can information about you still be used and/or collected?

Information that has already been gathered using your samples, will still be used anonymously for publication. However, your name shall not be released in reference to any information collected from you.

13c. Can the Principal Investigator withdraw you from this research study?

You may be withdrawn from the study without your consent for the following reasons:
Failure to be available when needed. Failure to turn in membrane when requested.

14. How will your privacy and the confidentiality of your research records be protected?

Authorized persons from the University of Florida, the hospital or clinic (if any) involved in this research, and the Institutional Review Board have the legal right to review your research records and will protect the confidentiality of them to the extent permitted by law. Otherwise, your research records will not be released without your consent unless required by law or a court order.

If the results of this research are published or presented at scientific meetings, your identity will not be disclosed.

15. How will the researcher(s) benefit from your being in this study?

In general, presenting research results helps the career of a scientist. Therefore, the Principal Investigator may benefit if the results of this study are presented at scientific meetings or in scientific journals.

16. Signatures

As a representative of this study, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternatives to being in the study; and how privacy will be protected:

____ ____________________

Signature of Person Obtaining Consent Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your privacy will be protected. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

You voluntarily agree to participate in this study. By signing this form, you are not waiving any of your legal rights.

____ ____________________

Signature of Person Consenting Date

APPENDIX B
GAS CHROMOTOGRAPHIC CHARTS OF CARBON STANDARDS AND SAMPLES FROM TRIAL 1 AND 2

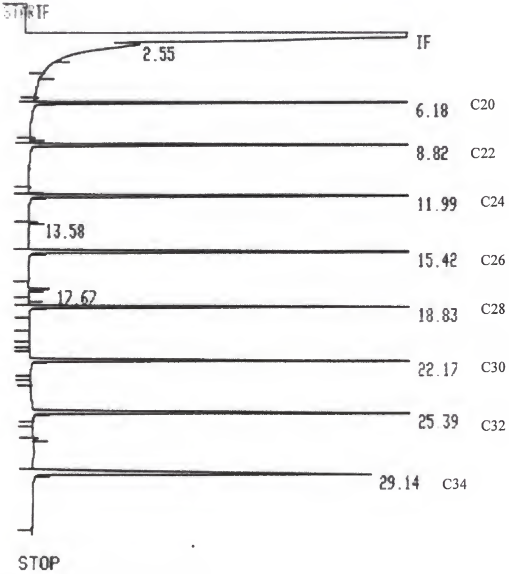


Figure B-1. Hydrocarbon standards

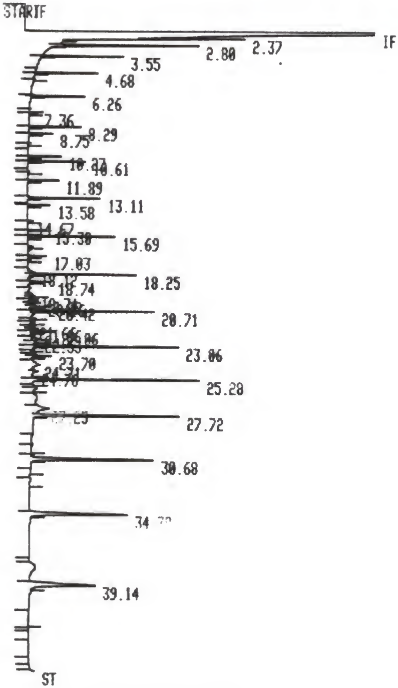


Figure B-2. Untreated sample from trial 1

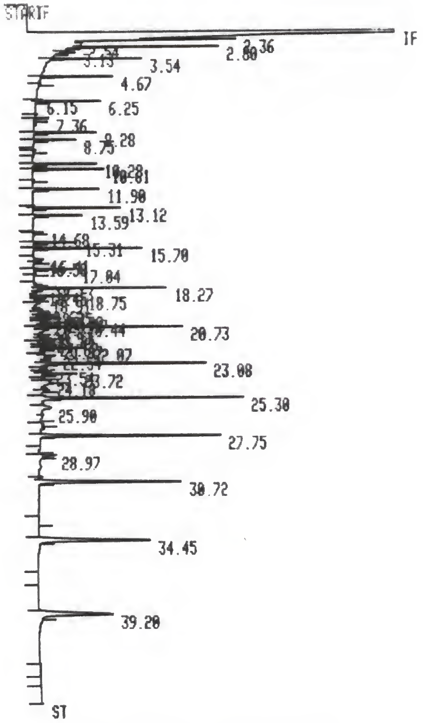


Figure B-3. Human subject 8005, sample from trial 1

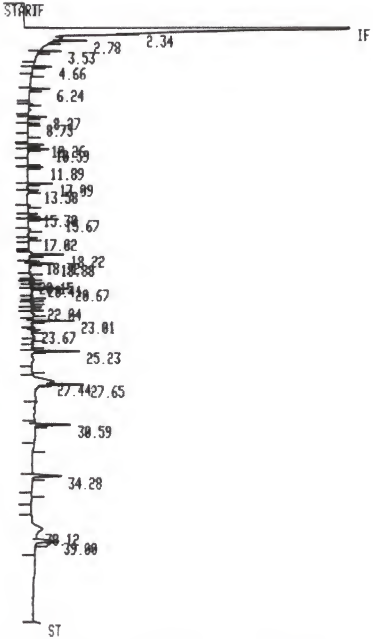


Figure B-4. Human subject 8007, sample from trial 1

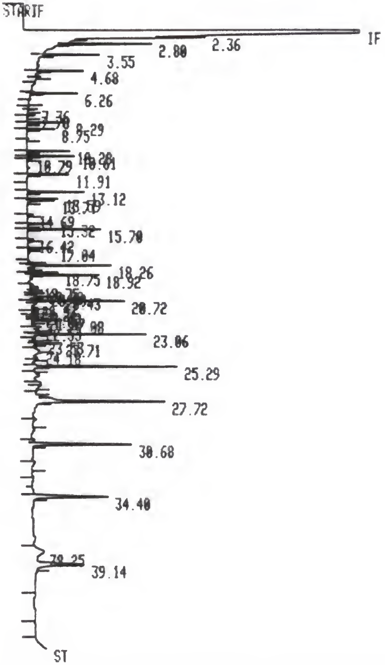


Figure B-5. Re-run of human subject 8007, sample from trial 1

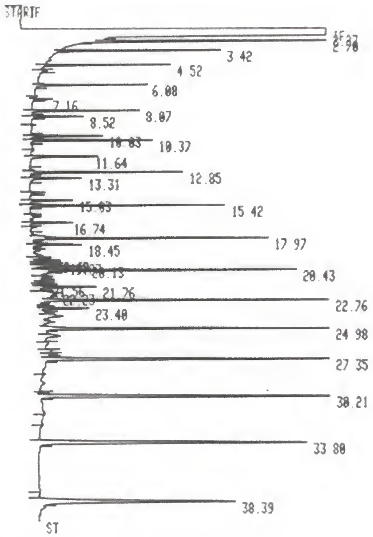


Figure B-6. Untreated sample from trial 2

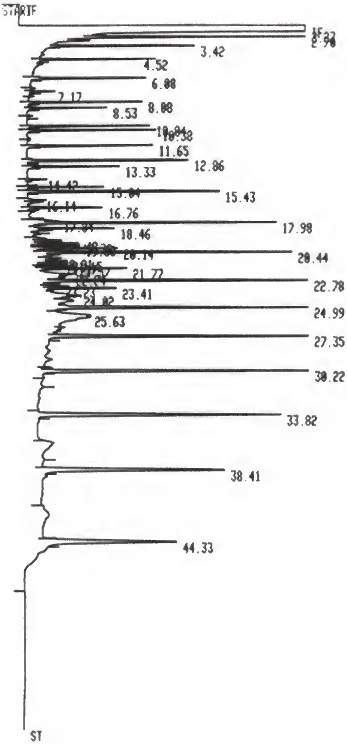


Figure B-7. Human subject 8005, sample from trial 2

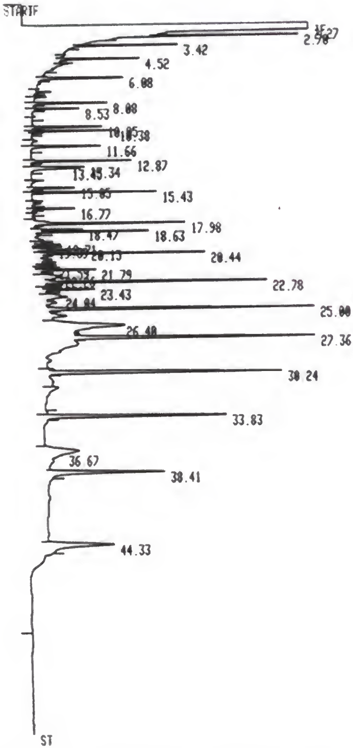


Figure B-8. Human subject 8007, sample from trial 2

APPENDIX C
GAS CHROMATOGRAPHIC TABLES

Table C-1.Temperature programmed gas chromatographic traces of Hydrocarbon standards table

Carbon #	RT (min)	Area	Type	AR/HT	Area %
C20	6.18	89421	PB	0.065	16.621
C22	8.82	77270	PB	0.078	14.362
C24	11.99	73798	PB	0.088	13.717
C26	15.42	74112	PB	0.101	13.775
C28	18.83	46631	PB	0.089	8.667
C30	22.17	49203	PB	0.094	9.145
C32	25.39	56780	PB	0.105	10.554
C34	29.14	68228	PB	0.160	12.682

Table C-2. Temperature programmed gas chromatographic traces of Standard (3776) trial 1

Peak #	RT (min)	Area %	RI
1	2.37	2.215	—
2	2.80	5.475	—
3	3.55	2.542	—
4	4.68	1.994	—
5	6.26	1.849	2006
6	7.36	0.336	2089
7	8.29	1.844	2160
8	8.75	0.912	2195
9	10.27	1.342	2291
10	10.61	2.167	2313
11	11.89	1.417	2394
12	13.11	2.843	2465
13	13.58	0.887	2493
14	14.67	0.281	2556
15	15.30	0.849	2593
16	15.69	3.551	2616
17	17.03	0.806	2694
18	18.12	0.408	2758
19	18.25	4.591	2766
20	18.74	0.802	2795
21	19.74	0.095	2854
22	19.99	0.541	2869
23	20.16	0.547	2880
24	20.42	1.240	2895
25	20.71	4.790	2913
26	21.66	0.321	2969
27	21.86	0.391	2981
28	22.06	1.319	2993
29	22.53	0.224	3022
30	23.06	6.073	3055
31	23.70	1.033	3095
32	24.31	1.281	3133
33	24.76	0.561	3161
34	25.28	7.535	3193
35	27.25	2.188	3299
36	27.72	8.682	3324
37	30.68	9.005	—
38	34.39	8.930	—
39	39.14	8.136	—

Table C-3. Temperature programmed gas chromatographic traces of Sample 8005, trial 1

Peak #	RT (min)	Area %	RI
1	2.36	2.654	—
2	2.54	0.961	—
3	2.80	4.073	—
4	3.13	0.349	—
5	3.54	2.041	—
6	4.67	1.685	—
7	6.15	0.158	—
8	6.25	1.639	2005
9	7.36	0.365	2089
10	8.28	1.627	2159
11	8.75	1.178	2195
12	10.28	1.857	2292
13	10.61	1.917	2313
14	11.90	1.961	2394
15	13.12	2.514	2466
16	13.59	1.424	2493
17	14.68	0.318	2557
18	15.31	1.309	2594
19	15.70	3.154	2616
20	16.41	0.359	2658
21	16.58	0.193	2668
22	17.04	1.222	2695
23	18.13	0.734	2759
24	18.27	4.446	2767
25	18.45	0.299	2778
26	18.75	1.441	2795
27	18.91	0.375	2805
28	19.62	0.339	2847
29	19.75	0.427	2855
30	20.00	0.847	2870
31	20.17	0.771	2880
32	20.35	0.297	2891
33	20.44	1.356	2896
34	20.73	4.306	2914
35	20.93	0.762	2926
36	21.21	0.347	2943
37	21.42	0.122	2955
38	21.67	0.513	2970
39	21.87	0.609	2982
40	22.07	1.779	2994
41	22.54	0.709	3023
42	23.08	5.411	3057
43	23.54	0.397	3085
44	23.72	1.325	3096
45	24.18	0.008	3125
46	25.30	6.829	3194
47	25.90	0.994	3227
48	27.75	7.621	3326
49	28.97	0.829	3391
50	30.72	7.888	—
51	34.45	7.997	—
52	39.20	7.264	—

Table C-4. Temperature programmed gas chromatographic traces of Sample 8007, trial 1

Peak #	RT (min)	Area %	RI
1	2.34	2.550	—
2	2.78	4.455	—
3	3.53	1.982	—
4	4.66	1.659	—
5	6.24	2.035	2005
6	8.27	1.819	2158
7	8.73	1.117	2193
8	10.26	1.746	2291
9	10.59	1.909	2312
10	11.89	1.785	2394
11	13.09	2.425	2464
12	13.58	1.212	2493
13	15.30	1.082	2593
14	15.67	3.044	2615
15	17.02	1.058	2694
16	18.22	4.086	2764
17	18.72	1.130	2794
18	18.88	2.803	2803
19	20.15	0.311	2879
20	20.41	0.723	2895
21	20.67	3.949	2910
22	22.04	1.472	2992
23	23.01	4.501	3052
24	23.67	1.157	3093
25	25.23	5.314	3190
26	27.44	8.418	3309
27	27.65	7.541	3321
28	30.59	6.348	—
29	34.28	6.991	—
30	38.12	7.829	—
31	39.00	7.551	—

Table C-5. Temperature programmed gas chromatographic traces of Sample 8007, trial 1, re-run

Peak #	RT (min)	Area %	RI
1	2.36	0.370	—
2	2.80	3.700	—
3	3.55	2.098	—
4	4.68	1.673	—
5	6.26	1.841	2006
6	7.36	0.415	2089
7	7.78	0.343	2121
8	8.29	1.905	2160
9	8.75	1.173	2195
10	10.28	1.843	2292
11	10.61	1.920	2313
12	10.79	0.304	2324
13	11.91	1.804	2395
14	13.12	2.577	2466
15	13.59	1.372	2493
16	13.71	1.479	2500
17	14.69	0.285	2557
18	15.32	1.230	2594
19	15.70	3.058	2616
20	16.42	0.205	2659
21	17.04	1.158	2695
22	18.26	3.896	2767
23	18.75	1.248	2795
24	18.92	3.103	2805
25	19.75	0.368	2855
26	20.00	0.775	2870
27	20.18	0.695	2881
28	20.43	1.583	2896
29	20.72	4.152	2913
30	20.93	0.626	2926
31	21.21	0.093	2943
32	21.42	0.520	2955
33	21.67	0.441	2970
34	21.87	0.573	2982
35	22.08	1.697	2995
36	22.53	0.504	3022
37	23.06	5.437	3055
38	23.53	0.494	3084
39	23.71	1.382	3096
40	24.18	0.000	3125
41	25.29	6.882	3194
42	27.72	9.392	3324
43	30.68	7.731	—
44	34.40	7.619	—
45	38.25	3.014	—
46	39.14	7.023	—

Table C-6. Temperature programmed gas chromatographic traces of Standard (3776), trial 2

Peak #	RT (min)	Area %	RI
1	2.27	0.000	—
2	2.70	5.763	—
3	3.42	2.752	—
4	4.52	2.157	—
5	6.08	2.137	—
6	7.16	0.377	2074
7	8.07	1.993	2143
8	8.52	0.954	2177
9	10.03	1.392	2276
10	10.37	2.272	2298
11	11.64	1.462	2378
12	12.85	3.010	2450
13	13.31	1.014	2477
14	15.03	0.863	2577
15	15.42	3.732	2600
16	16.74	0.812	2677
17	17.97	5.178	2750
18	18.45	0.878	2778
19	19.69	0.558	2851
20	19.87	0.519	2862
21	20.13	1.257	2878
22	20.43	4.862	2896
23	21.56	0.388	2963
24	21.76	1.307	2975
25	22.23	1.469	3004
26	22.76	6.400	3037
27	23.40	0.919	3076
28	24.98	8.420	3175
29	27.35	9.126	3305
30	30.21	9.495	—
31	33.80	9.778	—
32	38.39	8.759	—

Table C-7. Temperature programmed gas chromatographic traces of Sample 8005, trial 2

Peak #	RT (min)	Area %	RI
1	2.27	0.556	—
2	2.70	3.899	—
3	3.42	2.066	—
4	4.52	1.692	—
5	6.08	1.826	—
6	7.17	0.393	2075
7	8.08	1.722	2144
8	8.53	1.205	2178
9	10.04	1.940	2277
10	10.38	1.925	2298
11	11.65	2.033	2379
12	12.86	2.567	2451
13	13.33	1.451	2478
14	14.42	0.339	2542
15	15.04	1.264	2578
16	15.43	3.066	2601
17	16.14	0.326	2642
18	16.76	1.169	2679
19	17.84	0.546	2742
20	17.98	4.168	2750
21	18.46	1.309	2778
22	19.48	0.752	2839
23	19.70	0.719	2852
24	19.88	0.668	2863
25	20.14	1.499	2878
26	20.44	4.087	2896
27	20.91	0.714	2925
28	21.15	0.787	2939
29	21.37	0.542	2952
30	21.57	0.560	2964
31	21.77	1.704	2976
32	22.24	1.112	3004
33	22.78	5.164	3038
34	23.23	0.408	3066
35	23.41	1.210	3077
36	24.02	1.810	3115
37	24.99	6.279	3175
38	25.63	3.680	3213
39	27.35	6.429	3305
40	30.22	6.704	—
41	33.82	7.043	—
42	38.41	6.655	—
43	44.33	6.014	—

Table C-8. Temperature programmed gas chromatographic traces of Sample 8007, trial 2

Peak #	RT (min)	Area %	RI
1	2.27	0.000	—
2	2.70	2.888	—
3	3.42	1.776	—
4	4.52	1.391	—
5	6.08	1.666	—
6	8.08	1.604	2144
7	8.53	0.930	2178
8	10.05	1.475	2278
9	10.38	1.634	2298
10	11.66	1.636	2379
11	12.87	2.143	2451
12	13.34	1.061	2479
13	13.45	1.284	2485
14	15.05	1.015	2578
15	15.43	2.531	2601
16	16.77	0.875	2679
17	17.98	3.984	2750
18	18.47	0.988	2779
19	18.63	2.340	2788
20	19.71	0.526	2853
21	19.89	0.496	2863
22	20.15	1.287	2879
23	20.44	3.713	2896
24	21.59	0.456	2965
25	21.79	1.366	2977
26	22.26	0.327	3006
27	22.78	4.429	3038
28	23.43	1.005	3078
29	24.04	1.325	3116
30	25.00	6.841	3176
31	26.40	7.203	3254
32	27.36	7.819	3305
33	30.24	7.442	—
34	33.83	7.000	—
35	36.67	6.109	—
36	38.41	6.325	—
37	44.33	5.109	—

APPENDIX D
GAS CHROMOTOGRAPHIC GRAPH OF STEROL STANDARDS

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TOTAL AREA=        4971
MUL FACTOR= 1.0000E+00
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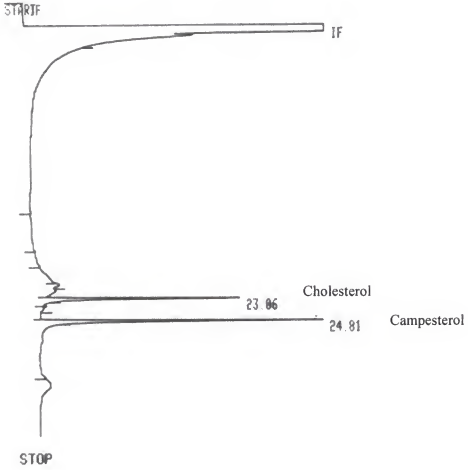

APPENDIX E
GAS CHROMATOGRAPHIC TABLES FOR STEROL STANDARDS

Table E-1. Temperature programmed gas chromatographic traces of cholesterol and campesterol, standard run 1

Peak #	RT (min)	Area %	RI	Name
1	23.13	34.661	3060	CHOLESTEROL
2	24.87	65.339	3168	CAMPESTEROL

Table E-2. Temperature programmed gas chromatographic traces of cholesterol and campesterol, standard run 2

Peak #	RT (min)	Area %	RI	Name
1	23.06	41.734	3055	CHOLESTEROL
2	24.81	58.266	3164	CAMPESTEROL

Table E-3. Temperature programmed gas chromatographic traces of Campesterol, standard run 3

Peak #	RT (min)	Area %	RI	Name
1	24.83	100	3165	CAMPESTEROL

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BIOGRAPHICAL SKETCH

Karen E. McKenzie was born on the 14th of October 1969 in Mobile, Alabama. She was kept in the hospital for four months after her birth until a loving family adopted her in January of 1970. David and Myrtle McKenzie raised their second adopted child in Wakulla County, a small rural community just outside of Florida's state capitol. Summer and after-school jobs at veterinary clinics nurtured an interest in diseases of animals, many of which were transmitted by insects. These jobs also served to support a part-time college career at Tallahassee Community College. After receiving an A.A. degree she transferred to the University of Florida, where she completed a Bachelor of Science degree in animal sciences and then followed that with another B.S. in entomology, in 1997. In May of 2000 she received a master's degree in medical and veterinary entomology. Since that time she has continued in the field of medical and veterinary entomology, working on a Ph.D.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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